Universidade de Lisboa

Faculdade de Farmácia

PRELIMINARY STUDY TOWARDS A NOVEL CHRONIC EXPERIMENTAL MODEL TO STUDY INFLAMMATORY BOWEL DISEASE

Rita Eusébio Modesto

Dissertation supervised by Professor Rui Manuel Amaro Pinto and co-supervised by Professor Vanessa Alexandra Pinho Mateus

Biopharmaceutical Sciences

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"Recomeça... se puderes, sem angústia e sem pressa e os passos que deres, nesse caminho duro do futuro, dá-os em liberdade, enquanto não alcances não descanses, de nenhum fruto queiras só metade. "

Miguel Torga

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Abstract

Inflammatory bowel disease is a chronic inflammatory disorder of the epithelium of the intestinal tract caused by multiple factors and for which therapeutic options are limited. IBD is an idiopathic disease and the exact cause remains unknown. Patients with IBD have increased intestinal permeability, barrier disfunction of the epithelium and cumulative exposure to antigens leading to activation of the immune system, production of pro-inflammatory cytokines and reactive oxygen species resultant in intestinal mucosa lesions. The treatment currently used to IBD requires a long-term pharmacological approach based on the combination of drugs that have anti-inflammatory activity and allows the inhibition of the initial events of inflammation, however consist only of symptomatic and palliative treatments and are unable to maintain remission of inflammation in the colon for long periods of time. Experimental models of IBD supply considerable information on the pathogenesis of this illness and represent an important tool in testing new strategies of treatment, since they mimic the features of the disease. The main aim of this project is to develop a preliminary study towards a novel chronic experimental model to study IBD in order to support a future new model of chronic induced colitis in rodents using TNBS. The experimental model with TNBS consists in the induction of intestinal inflammation by a chemical process through intracolonic administration of TNBS. The mice were evaluated taking into account parameters such as body weight, stool consistency, anus appearance and colon length and biochemical markers such as ALP, urea, creatinine, ALT, fecal hemoglobin, TNF-α, IL-10 and histopathological score. TNBS-induced chronic colitis was tested in 6 weeks, with weekly TNBS instillations, providing a chronic intestinal inflammation model. The TNBS groups showed a slight change in intestinal motility characterized by diarrhea, anus edema and moderate morbidity, while the control groups remained unchanged. These mice also showed a slight and progressive increase in body weight, a reduced colon length, and an increase in fecal hemoglobin concentration, which was maintained until the end of the experimental protocol. Colitic mice presented raised values of ALP, TNF-α and fecal hemoglobin after week 4 until the end of the experiment, and contrary to expected increase values of an anti-inflammatory cytokine, IL-10. These results are consistent with the correct induction of experimental chronic colitis by TNBS. These preliminary data allow suggesting that TNBS-induced chronic colitis should be developed in 4 weeks, providing a chronic intestinal inflammation model.

Key-Words: Inflammatory Bowel Disease, Crohn Disease, Ulcerative Colitis, TNBS-induced colitis, animal model

Resumo

A doença inflamatória intestinal (DII) é uma desordem inflamatória crónica do epitélio do trato intestinal causada por múltiplos fatores e para os quais as opções terapêuticas são limitadas [1,2]. A DII é uma doença idiopática na qual a causa exata permanece desconhecida [3]. Os pacientes com DII apresentam permeabilidade intestinal aumentada, disfunção da barreira do epitélio e exposição cumulativa a antigénios que levam à ativação do sistema imunitário, produção de citocinas pro-inflamatórias e espécies reativas de oxigénio resultando em lesões da mucosa intestinal [4]. O tratamento atualmente utilizado para DII requer uma abordagem farmacológica de longo prazo baseada na combinação de drogas que têm atividade anti-inflamatória e permite a inibição dos eventos iniciais de inflamação, no entanto consiste apenas em tratamentos sintomáticos e paliativos que são incapazes de manter a remissão da inflamação no cólon por longos períodos de tempo [4,5]. Os modelos experimentais de doença inflamatória intestinal fornecem informações consideráveis sobre a patogénese da doença e representam uma ferramenta importante no teste de novas estratégias de tratamento [6,7]. O principal objetivo deste projeto é desenvolver um estudo preliminar em direção a um novo modelo experimental crónico para estudar a DII, a fim de apoiar um futuro modelo de colite crónica em roedores com TNBS. O novo modelo experimental de colite crónica deve ser capaz de simular a doença inflamatória intestinal em humanos, para posterior estudo das vias metabólicas envolvidas na DII e futura modulação farmacológica com o objetivo de melhorar os tratamentos para DII atualmente conhecidos.

O modelo experimental com TNBS consiste na indução de inflamação intestinal por um processo químico através da administração intracolónica de TNBS como descrito pelo método de Morris [8]. Utilizou-se murganhos CD-1 de 6-10 semanas de idade e com 25-40g de peso. Os grupos experimentais foram estruturados de acordo com o principal objetivo do estudo. Foram ainda formados grupos de controlo para servirem de referência na comparação dos resultados com os grupos em estudo. No final do período experimental, os ratos foram anestesiados e foram recolhidas amostras de sangue por punção cardíaca. Em seguida, os murganhos foram sacrificados por deslocação cervical e necropsiados. Os murganhos foram avaliados tendo em conta sinais clínicos como peso corporal, consistência das fezes, aparência do ânus e comprimento do cólon e marcadores bioquímicos como ALP, ureia, creatinina, ALT, hemoglobina fecal avaliados através do método automático ADVIA e TNF-α e IL-10 determinados por ELISA. O *score* histopatológico final será avaliado com base na gravidade e extensão das lesões segundo os critérios adaptados de Corazza e colegas [9] e Seamons e colegas [10]. As seções histopatológicas serão examinadas e pontuadas de acordo com a presença de inflamação (0-4 de gravidade crescente) com alguns parâmetros, a saber: (1) presença de perda / necrose tecidual, (2) gravidade da lesão epitelial da mucosa, (3) inflamação, (4) extensão 1 - a percentagem de intestino afetado de qualquer maneira e (5) extensão 2 - a percentagem de intestino afetado pela lesão mais grave. A gravidade da colite foi calculada somando as lesões individuais e os *scores* de extensão, promovendo um score final de colite (*score* máximo = 20).

O principal objetivo é o desenvolvimento e padronização do modelo de colite crónica induzida por TNBS, uma vez que a evolução de parâmetros associados a infeção crónica como da lesão intestinal mais grave causada pelo TNBS ocorrem gradualmente após uma semana de indução com durabilidade de até 8 semanas e os protocolos do modelo de colite induzida por TNBS não são padronizados [8,11,12]. Seis grupos independentes de colite induzida por TNBS em murganhos foram então monitorizados sob as mesmas condições específicas durante 6 semanas. Os grupos foram induzidos semanalmente e sacrificados no dia 7 de cada semana, dependendo do grupo experimental, a fim de se obter um modelo crónico de colite induzida por TNBS. O objetivo era identificar em que período de tempo o padrão de lesão crónica intestinal máxima se mantinha, pelo método de indução usado no presente estudo. Relativamente aos resultados obtidos nesta experiência, verificou-se que o modelo crónico preliminar de colite induzida por TNBS desenvolveu-se a partir da quarta semana de indução. O grupo TNBS apresentou uma leve alteração da motilidade intestinal caracterizada por diarreia, edema do ânus e morbilidade moderada, enquanto os grupos controlo permaneceram sem alterações. Estes murganhos apresentaram ainda um aumento leve e progressivo do peso corporal, um comprimento do cólon reduzido e um aumento da concentração de hemoglobina fecal, que se mantém até ao final do protocolo experimental. A indução de colite por TNBS promoveu, ao fim de 4 semanas, um aumento dos mediadores inflamatórios, tais como, TNF-α, e contrariamente ao esperado aumento da uma citocina anti-inflamatória, IL-10, bem como a manutenção destes valores até ao final do período experimental. Estes resultados são consistentes com a indução correta de colite crónica experimental por TNBS.

Em conclusão, a validação desse modelo animal é realmente relevante para a comunidade científica, pois, até á data, não existe prática padronizada na indução de colite por TNBS e a utilização deste modelo em futura modulação farmacológica pode facilitar um tratamento mais eficaz e seletivo do que o atualmente conhecido.

Palavras-chave: Doenças Inflamatórias Intestinais, Doença de Crohn, Colite Ulcerativa, Colite induzida por TNBS, Modelo animal

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Acronyms and Abbreviations

5-ASA – 5-Aminosalicylic acid
ALP - Alkaline phosphatase
ALT - Alanine aminotransferase
Anti-TNF-α - Monoclonal antibodies against TNF-α
CD - Crohn's disease
CDAI - Crohn's disease activity index
COX - Cyclooxygenase
DSS - Dextran sulfate sodium
EPO - Erythropoietin
GM-CSF - Granulocyte macrophage colony stimulating factor
IBD - Inflammatory bowel diseases
IFN-γ - Interferon-γ
IκB - Inhibitory κB
IL - Interleukin
IP - Intraperitoneal injection
IV - Intravenous
NaCl - Sodium chloride
NF-κB - Nuclear transcription factor kappa B
ROS - Reactive oxygen species
STAT - Signal transducer and activator of transcription protein
Th - T helper
TDZD-8 - Thiadiazolidinone-8
TNBS - Trinitrobenzene sulfonic acid
TNF-α - Tumor necrosis factor α
UC - Ulcerative colitis

Introduction

Inflammatory bowel disease (IBD) is a common chronic inflammatory disease of the gastro-intestinal tract characterized by chronic inflammation of the intestinal epithelium, bowels ulceration and damaging mucosal tissues, resulting in symptoms of diarrhea, weight loss and abdominal pain [1,2]. IBD results from inappropriate and uncontrolled immune responses driven by the luminal flora in genetically susceptible individuals. Crohn's disease (CD) and Ulcerative colitis (UC) are the two major forms of IBD and can be distinguished by the location of inflammation within the gastrointestinal tract [5]. These disorders present similarity, however, they are distinguished by the different pathophysiological aspects and clinical manifestations [4]. CD presents transmural inflammation in the entire length of the GI tract, although it predominantly affects the region adjacent to the ileum and cecum [13]. Typically presents in a discontinuous manner, affecting several proportions of the gastrointestinal tract and associated with complications such as fistulas, stenosis and abscesses [14]. In contrast, UC patients, inflammation usually involves the rectum and extends contiguously in the colon by a variable degree affecting only the superficial mucosa [14].

The IBD is a chronic and intermittent disease, during relapses, symptoms severity varies between slight to severe and during remissions the symptoms can even diminish or disappear. Generally, symptoms depend on the intestinal affected segment and usually include diarrhea often with blood, colic abdominal pain and fecal urgency. Beyond these, other unspecific symptoms may occur like fever, loss of appetite and weight, fatigue and primary amenorrhea [15].

Immunologically patients with IBD have continuously activated innate and acquired immune system with increased recruitment and retention of effector macrophages, neutrophils, T and B cell in the local inflammation where they are activated, release pro-inflammatory cytokines, and loss of tolerance to enteric commensal bacteria. The higher recruitment as well as prolonged survival of effector cells caused by decreased cellular apoptosis exacerbate the immune response and promote the accumulation of effector cells in local intestinal area [16]. Thus, pathogenesis of inflammatory bowel disease is not completely understood but, it is well accepted that abnormal mucosal immune response, microbial factors and epithelial cell abnormalities can facilitate this response [17].

Otherwise, the histologic involvement of lymphocytes T helper CD4 is observed in both subtypes of IBD, however the pro-inflammatory cytokines responsible for the establishment and development of pathology are different [4]. The inflammatory response in CD is mediated by lymphocytes Th1 and Th17 with high levels of interleukin 12 (IL-12), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) [3,4,16]. By his side, UC inflammatory response is atypically unleashed by lymphocytes Th2, increasing interleukins 4 and 5 and granulocyte macrophage colony stimulating factor.

The IBD has been revealed a world health problem with growing incidence, particularly in subjects with 15 to 30 years old [4]. The IBD has increased incidence in Portugal, currently affecting around 7000 to 15000 Portuguese [18]. Statistical data show a marked increase in the countries of the southern hemisphere, although it is still more prevalent in north hemisphere countries, which can be justified by the lifestyle westernization of habitants from poorest countries [4]. Specifically, the hygiene hypothesis reveals that persons less exposed in childhood to infections or unsanitary conditions lose commensal organisms or organisms that regulates T cell development, or instead do not develop a sufficient immune repertoire once they were not exposed to necessary noxious organisms. Such individuals are associated with a higher incidence of chronic immune diseases, including inflammatory bowel disease [19,20,21].

The etiology of IBD remains undefined, but it is well accepted that IBD is multifactorial, involving interactions between host immune system, genetic susceptibility and responses to environmental and microbial factors [17]. Genetic and environmental factors, immune system dysregulation, modification of luminal bacteria and the increase of intestinal permeability, have a crucial role in the dysregulation of intestinal immunity, origin gastrointestinal injury. [7,22] Four genes have been associated with Crohn's disease, CARD15, SLC22A4/5, DLG5, PPARG and one with ulcerative colitis, MDR1, it is suggested that these genes regulate several important biologic functions, including immune regulation, mucosal barrier integrity and microbial homeostasis [7,16]. Several environmental factors like smoking, diet, the use of antibiotics and non-steroidal anti-inflammatory drugs, stress and infection are proposed as possible cause in the pathogenesis of inflammatory bowel disease [16,23,24]. These factors trigger in patients with IBD increased intestinal permeability, which leads to a decrease in the barrier function of the epithelium, allowing greater exposure to antigens and consequent activation of the immune system and pro-inflammatory cytokines raising inflammation [16].

The diagnostic of CD and UC includes clinical characteristics, laboratorial tests like complete blood count, sedimentation rate, biochemical analyzes such as c-reactive protein, ferritin, serum albumin, fecal occult blood test and stool culture to mislead infections caused by microorganisms. More specifically, is used Anti-Saccharomyces cerevisiae antibodies (ASCA) and Perinuclear Anti-Neutrophil Cytoplasmic Antibodies (p-ANCA) which allow diagnostic precision in complex cases and distinguish CD from UC. Besides that, abdominal X-ray, colonoscopy, endoscopy and biopsy should be done. [1]

The treatment of IBD often requires a long-term pharmacological approach based on the combination of drugs to control acute reactions, maintain remission and treat specific complications. Actually, therapeutics to the treatment of IBD include a large number of drugs, like aminosalicylates, glucocorticoids, immunosuppressants, immunomodulators and biologic therapy, which have little selectivity for IBD [4,5]. The majority of therapeutics have anti-inflammatory activity that allows the inhibition of the initial events of inflammation with the migration of inflammatory mediators, vasodilation, vascular permeability and leukocyte infiltration. These drugs also act decreasing macrophages recruitment, inhibit the production of IL-1 by monocytes, IL-2 and TNF-α by lymphocytes, phospholipase A2, prostaglandins and leukotrienes [4,5].

Unfortunately, the therapeutic options available today do not bring alive the cure to IBD and the accessible treatments have serious side effects and high cost, besides that, there are patients who do not respond to any of the treatments, aspects that greatly limit its use [5]

Nowadays IBD treatments introduce risks to patients, do not represent the cure for the disease, consist only of symptomatic and palliative treatments and are unable to maintain remission of inflammation in the colon for long periods of time, indicating the urgent need to search for new products with intestinal anti-inflammatory activity for cure or prevent this disease [1,5].

In our research group, we have tested several new pharmacological approaches with benefits in this field [25-30], in this sense it is extremely important clarify the inflammatory process related with this pathology to propose new strategies to modulate inflammatory response, different from those already known, to treat effectively IBD and carrying out further studies in this and other areas that may solve many of the puzzles that mark this pathology.

With this project we propose to develop a preliminary study towards a novel chronic experimental model to study IBD, able to mimic inflammatory bowel disease in humans, for posterior study metabolic pathways involved in IBD and future pharmacological modulation. Thus, this thesis was divided into two chapters of literature review, one chapter of methodology, one chapter of presentation and discussion of results related to the development of the model, one chapter of the final discussion and main conclusions and, finally, one last chapter of the references. Therefore:

- Chapter I: state of the art description of inflammatory bowel disease, including its classification, diagnosis, as well as pharmacological treatment currently recommended;
- Chapter II: relevance of studies in animal models, presentation of existing animal models of experimental colitis and characterization of the chemically induced model of colitis used in this experimental work;
- Chapter III: description of the used methodology, such as the induction method,
experimental groups, evaluated parameters and statistical tests;
- Chapter IV: presentation of the results of the development of experimental colitis induced by TNBS;
- Chapter V: discussion of the development of experimental colitis induced by TNBS;
- Chapter VI: synthesis, discussion and the main conclusions of the work, as well as future prospects;
- Chapter VII: all references used in the development of the thesis.

Aim

The main aim of this project is to develop a preliminary study towards a novel chronic experimental model to study IBD in order to support a future new model of chronic induced colitis in rodents using TNBS. The model should be able to mimic inflammatory bowel disease in humans, for posterior test a set of drugs that modulate some important metabolic pathways in the establishment and development of inflammation in IBD.

Chapter 1 - Inflammatory Bowel Disease

1. Definition

IBD is a chronic, idiopathic, and relapsing inflammatory disease that affect the gastrointestinal tract through a cascade of inflammatory reactions that culminate in an immune system response. IBD is characterized by chronic inflammation of the intestinal epithelium, bowels ulceration and damaging mucosal tissues, resulting in symptoms like diarrhea, weight loss and abdominal pain [1,2].

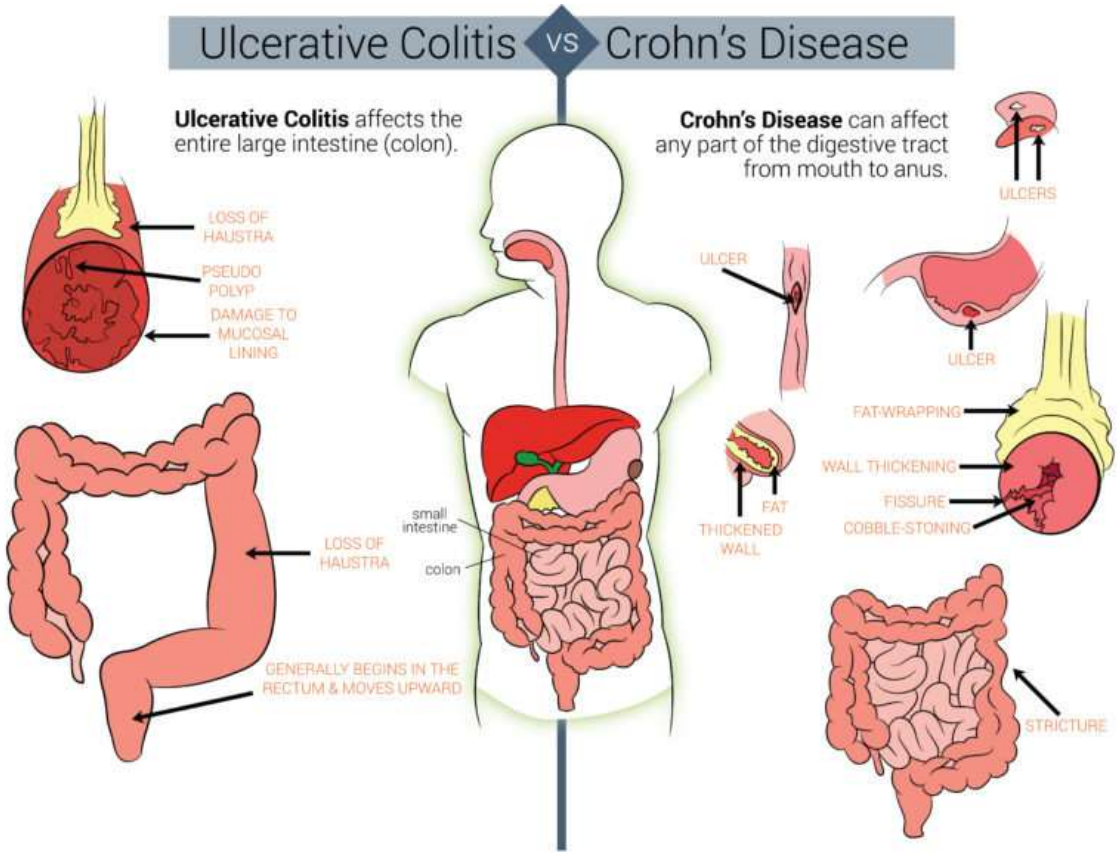


Figure 1. Ulcerative Colitis vs Crohn´s Disease [adapted from site QuIBD.com]

UC and CD are the two major forms of IBD, have different pathological and clinical characteristics, in which they differ in the extent and distribution of inflammation in the gastrointestinal tract and in the depth of involvement of the intestinal wall (Figure 1) [4,5]. Ulcerative colitis is a recurrent chronic ulcer inflammatory disorder that begins in the rectum and extends contiguously along the colon in the retrograde direction with inflammatory involvement by a variable degree affecting the inner layer of the mucosa, with diffuse and uniform inflammation. It is a disease in continuity and has as characteristics the presence of abdominal pain and episodes of bloody mucoid diarrhea. The course of UC is characterized by symptomatic periods interspersed with periods of remission [31,32].

In contrast, the CD lesions are distributed in a non-confluent pattern, presenting transmural inflammation, reaching all layers of the digestive wall. This disease can affect the entire length of the GI tract, from the mouth to the anus, although it predominantly affects the region adjacent to the ileum and cecum, altering the entire intestinal integrity [13,33,34]. The progression of the disease is usually characterized by episodes of exacerbation and remission of variable duration. The clinical presentation will depend on the location of the disease, including diarrhea, abdominal pain, fever, signs of intestinal obstruction, presence of blood and mucus in the feces and perianal lesions. CD is associated with complications such as fistulas, stenosis, abscesses and intestinal obstruction (Table 1) [35-37].

A third classification of IBD, IBD unclassified or indeterminate colitis, can be used when a distinction in diagnosis of UC and CD cannot be made through clinical signs and when all diagnostic exams where take in consideration. However, indeterminate colitis only represents a minority of cases of IBD, thus allowing in the majority of cases monitoring and treatment of the disease [34].

Table 1. Differences between Crohn´s disease and ulcerative colitis

[adapted from MSD Professional Edition Manuals, 2019]

Crohn's Disease	**Ulcerative Colitis**
Small intestine involved in 80% of the time.	Disease is confined to the colon.
Retossigmoid is generally spared; the colonic involvement is usually on the right side.	Retosigmoid is invariably affected; the colonic involvement is generally on the left.
Severe rectal bleeding is uncommon, except in 75 to 85% of cases of Crohn's colitis.	Significant rectal bleeding is always gift.
Fistulas, masses, and abscesses are common.	No fistulas occur.
Perianal lesions are significant in 25 to 35% of cases.	No significant perianal lesions ever occur.
In radiographs, the intestinal wall is asymmetrically affected and in a segmental way, with areas spared between segments affected.	The intestinal wall is affected symmetrically and of the proximal rectum.
The endoscopic appearance is irregular, with discrete ulcerations separated by segments of normal-appearing mucosa.	The inflammation is uniform and diffuse.
Microscopic inflammation and fissures transmural; the distribution of lesions is usually highly focal.	Inflammation is restricted to the mucosa, except in severe cases.
Epithelioid granulomas (sarcoidosis type) are detected in the intestinal wall or lymph nodes in 25 to 50% of cases (pathognomonic).	No typical epithelial granulomas occur.

1.1 Classification

The current IBD classification, known as the Montreal Classification, resulted from the review of the Vienna classification, permits characterizes the clinical features of this disorders as well as, clarify the diagnosis of CD an UC [38,39].

The Montreal classification, regarding CD, considers 3 parameters, age of onset (A), location of disease (L) and disease behavior (B). The presentation of CD is highly variable. A single episode may not be followed by further episodes or the patient may experience continuous, unremitting disease (Table 2) [38,39].

Table 2. Montreal classification of Crohn`s disease

[adapted from Satsangi et al., 2006]

AGE AT DIAGNOSIS	A1	< 16 years
	A2	17 - 40 years
	A3	> 40 years
LOCATION	L1	Ileal
	L2	Colonic
	L3	Ileocolonic
	L4	Isolated upper disease
BEHAVIOUR	B1	Non-stricturing, non-penetrating
	B2	Stricturing
	B3	Penetrating
	P	Perianal disease modifier**

Since there is an inherent subjectivity to symptoms that may condition the evaluation of CD activity, there are several indices which are used as an aid in identifying the phase of the pathology. An additional tool, known as CDAI (Crohn's disease activity index), should be considered in patients with CD. CDAI is not a classification of CD but represents a numerical estimate of the interpretation of the patient's symptoms, allowing to define the severity of disease activity. The CDAI calculation is the sum of the products of a list of eight items, multiplied by weighting factors, the total of which defines the severity of disease activity. Thus, CD activity can be classified as mild to moderate, moderate to severe and severe to fulminant. Total values less than or equal to 150 are associated with quiescent or remission. Values above 150 are indicative of active disease, if greater than 450 allow the classification of extremely severe disease (Table 3) [34,40].

Table 3. Grading of disease activity in Crohn´s disease

[adapted from Dignass et al., 2012]

Mild	CDAI of 150 – 220 (eg. ambulatory, eating and drinking, < 10% weight loss)	No features of obstruction, fever, dehydration, abdominal mass or tenderness	CRP increased above the upper limit of normal
Moderate	CDAI of 220 – 450 (eg. intermittent vomiting or weight loss > 10%)	Treatment for mild disease ineffective or tender mass. No overt obstruction	CRP elevated above the upper limit of normal
Severe	CDAI > 450 (eg. cachexia (BMI < 18 Kg m^{-2}) or evidence of obstruction or abscess)	Persistent symptoms despite intensive treatment	CRP increased

Legend: CDAI - Crohn's Disease Activity Index; BMI - Body mass index; CRP - C reactive protein.

The typical follow-up of UC consists in periods of exacerbation alternating with periods of symptomatic remission. The determination of the clinical picture and subsequent individualization of the therapy depend of two factors: extent and severity of inflammation. The Montreal classification, relative to UC, assessed the disease according this two parameters, severity and extension. [38,39].

The Montreal classification of disease extent of UC allows extent to be defined into three subgroups (Table 4), according to the maximal extent of inflammation observed at colonoscopy. Extension can be divided into proctitis when only the rectum is inflamed, distal when the affected extension is up to the splenic, encompassing proctitis when it extends beyond the splenic angle not reaching the blind and pancolitis when it reaches the full extent to the blind [39].

Table 4. Montreal classification for ulcerative colitis extent

[adapted from Satsangi et al., 2006]

Extent	Anatomy
Ulcerative proctitis	Involvement limited to the rectum (that is, proximal extent of inflammation is distal to the rectosigmoid junction)
Left sided UC (distal UC)	Involvement limited to a proportion of the colorectum distal to the splenic flexure
Extensive UC (pancolitis)	Involvement extends proximal to the splenic flexure

Legend: UC – Ulcerative colitis.

UC is an intermittent and relapsing disease where patients experience periods with no symptoms, which makes diagnosis difficult since the symptoms are not sufficiently clear [31,32,41]. Hereupon, the Montreal classification also classified the UC severity as mild, moderate and severe and fulminant determined by clinical signs and symptoms (Table 5) [38]. The arbitrary distinction between the different classifications are determined by the clinical signs and symptoms and are, generally, clinical recommendations for therapeutic decision-making.

Table 5. Montreal classification for ulcerative colitis severity

[adapted from Satsangi et al., 2006]

	Mild	Moderate	Severe
BLOODY STOOLS/DAY	< 4	4 or more if	≥ 6 and
PULSE	< 90 bpm	≤ 90 bpm	> 90 bpm or
TEMPERATURE	< 37.5˚C	≤ 37.8˚C	> 37.8˚C or
HAEMOGLOBIN	> 11.5 g/dl	≥ 10.5 g/dl	< 10.5 g/dl or
ESR	< 20 mm/h	≤ 30 mm/h	> 30 mm/h or
CRP	Normal	≤ 30 mg/L	> 30 mg/L

Legend: ESR - Erythrocyte sedimentation rate; CRP - C reactive protein.

1.2 Epidemiology

The inflammatory bowel disease is a chronic disease that affects approximately 3 million people in Europe and has been revealed a world health problem with growing incidence [42]. IBD affects individuals of any age, however, it has two peaks of greater incidence, a first between 15 and 30 years and a second between 50 and 70 years [14,43] The annual healthcare costs were estimated at around 4.5–5.6 billion euros, in Europe, and 6.3 billion dollars, in the United States of America [44].

In the past, it was an uncommon condition, increasing its incidence in the developed countries, in particular in the Europe, Australia, New Zealand and North America, where it is estimated that its prevalence practically double over the next decade. The distribution of the disease has undergone changes, in particular in the over last 20 years, and has also expanded to developing countries [44].

Statistical data show a marked increase in the countries of the southern hemisphere, although it is still more prevalent in north hemisphere countries, which can be justified by the lifestyle westernization of habitants from poorest countries [4,45]. The hygiene hypothesis, a hypothetical explanation to this data, suggests that persons less exposed in childhood to infections or unhygienic conditions lose commensal organisms or organisms that regulates T cell development, or yet do not develop a sufficient immune repertoire once they were not exposed to essential harmful organisms, propose a higher incidence of chronic immune diseases, including inflammatory bowel disease, for that individuals [19-21]. These results may vary according to the study carried out,

since the epidemiological of IBD is limited due to the difficulty in carrying out population studies, the non-use of universally accepted diagnostic criteria and the scarcity of registries. However, it is notorious that the incidence of IBD has increased worldwide [18].

In Portugal, the IBD incidence has increased, currently affecting around 7000 to 15000 Portuguese however, epidemiological data are scarce, which does not allow the incidence and prevalence of IBD in our country over time. One study conducted between 2003 and 2007, which assessed the prevalence of IBD in Portugal, states that there was an increase from 86 to 146 per 100,000 people and that this increase was homogeneous in all districts of the country. The prevalence of UC and CD is similar, as his distribution pattern by age and sex, although female prevalence is slightly higher than the male sex. Particularly the districts of Lisbon and Porto were the most affected with the disease [18,44]. Furthermore, hospitalization rates have increased by 33% and 13%, concerning CD and UC patients, respectively, from 2000 to 2015 [44].

1.3 Etiology

The etiology of IBD remains undefined, but it is well accepted that IBD is multifactorial, involving interactions between host immune system, genetic susceptibility and responses to environmental and microbial factors. None of the factors alone is sufficient to the development of the disease [7,22]. Thus, the interaction between genetic susceptibility and an abnormal immune response with deregulation of the innate and adaptive immune response and commitment of the function of the epithelial barrier are the main cause to IBD (Figure 2) [14].

1.3.1 Environmental factors

Several environmental factors like smoking, diet, the use of antibiotics and non-steroidal anti-inflammatory drugs, stress and infection are proposed as possible cause in the pathogenesis of inflammatory bowel disease [16,23,24]. These factors trigger in patients with IBD increased intestinal permeability, which leads to a decrease in the barrier function of the epithelium, allowing greater exposure to antigens and consequent activation of the immune system and pro-inflammatory cytokines, raising inflammation, how-ever the mechanism on which this factors act promising the disease is not yet known [16].

IBD has a higher incidence in industrialized countries proposed due to the westernization of its lifestyle. Regions like Eastern Europe and Asia, where this increase in IBD can be observed, have recently adopted westernized diets, like low-fiber, high-sugar and high animal fat, suggesting the influence of diet in the development of the disease. Thus, it is logical to consider diet as a determinant factor in IBD since it is an enteric disease and it is known the ability of some foods to act as antigens promoting inflammation, such as alcohol, refined sugar and coffee and in turn others are preventive in the intestinal inflammatory process like fruits, vegetables and fibers, otherwise various data try to compose the relationship of IBD with a specific diet or food without success [46,47].

Since it is more common in developed areas and there is currently an increase in non-industrialized countries, has been developed a theory to explain the phenomenon, the hygiene hypothesis reveals that persons less exposed in childhood to infections or unsanitary conditions lose commensal organisms or organisms that regulates T cell development, or instead do not develop a sufficient immune repertoire, once they was not exposed to necessary noxious organisms, contributing to an immature immune system which causes an inappropriate immune response when exposure to noxious agents reoccurs [19,20].

Smoking is recognized as contributor to IBD, nicotine, carbon monoxide and hypoxia have all been suggested to be mediators of the effects of smoking on IBD [48-50]. However, smoking have different effects when it comes to CD and UC, if in turn the CD has an exacerbating effect on the disease in UC have an protective effect, nevertheless, there is still no explanation for this event [16]. CD patients who smoke tent to have more complications and more severe symptoms throughout the disease, indicative of detrimental effect on CD [47,51,52]. Contrary on UC smoking seems to have protective effect delay the beginning of disease and studies have shown that current smoking reduced the risk of UC [43,47].

Oral contraceptives and non-steroidal anti-inflammatory drugs are drugs studied as possible etiological factors with IBD. In the case of oral contraceptives their association with IBD is not yet fully known but it is believed that thrombogenic properties may be related to ischemia and changes in mucosal integrity and consequently allow the entry of antigens into the intestinal lumen [47]. Non-steroidal anti-inflammatory drugs in turn have a side effect causing injury in the gastrointestinal tract. There is an increase in vascular permeability and local lesion caused by these drugs and in addition a systemic effect on the prostaglandins, reducing the protection of the mucosa of the gastrointestinal tract causing alterations in the permeability of the colon membrane leading to the development of IBD [53].

Nowadays various changes in human behavior have been associated with an increase in the incidence of IBD. This increase in countries that have been to adopt an industrialized lifestyle suggests that the environmental factors are crucial as triggers of disease. There are epidemiological data that appear to show a close correlation between the infectious diseases, vaccination and stress of the population with increasing incidence of autoimmune diseases and chronic inflammatory diseases, but so far without conclusive results [47].

1.3.2 Genetic factors

Actually, there is evidence that different genetic factors, through different inflammatory mechanisms predispose to the development of IBD. The role of genetics in the pathogenesis of IBD was initially suggested in household and in twins, however there is no evidence of classic heredity. IBD is overlapping in both genders but occurrence of disease is suggested to be influenced by race and some specific ethnic groups [43,51,52].

At least 201 loci were identified, most of them associated with both forms, indicating common mechanisms in both diseases, but specific to CD and specific UC. Despite the divergence in the effect in the immune system, putative genes can be divided into those that influence innate immunity, autophagy, the epithelial barrier, immune responses, adaptive responses to oxidative stress and antimicrobial [7,16].

Four genes have been associated with Crohn's disease, CARD15, SLC22A4/5, DLG5, PPARG and one with ulcerative colitis, MDR1, it is suggested that these genes regulate several important biologic functions, including immune regulation, mucosal barrier integrity and microbial homeostasis [7,16]. CARD15 localized at chromosome 16 promotes NF-κB activation and regulation, killing of intracellular pathogens, paneth-cell function, α-defensin production and plays an important role in the innate immune response, apoptosis and recognition, conferring greater susceptibility to CD. SLC22A4/5 located at 5 chromosome has as function organic cation, carnitine transporters, possibly transport xenobiotic substances. The gene DLG5 in 10 chromosome is an epithelial scaffolding protein and PPARγ in chromosome 3 acts as an intracellular inhibitor of NF-κB and in cellular activation, lastly MDR1 located at 7 chromosome is an efflux transporter for drugs and, possibly, xenobiotic compounds. Other genes involved in the autogenic innate immune system, such as ATG16L1, IRGM and LRRK, and the genes involved in adaptive immunity, such as IL23R, IL12B and STAT3, are strongly associated with CD and possibly UC [7,16].

Genetic factors play a significant role in predisposition to IBD once increase susceptibility to IBD but also influence the degree of severity and extension of pathology. Genetic factors may be important for the understanding of the disease as well as the association of these with changes in the intestinal immune system are critical points for the knowledge of the physiology of the disease and for the development of new drugs [7,16].

1.3.3 Immunological Factors

The intestinal immune system is one of the largest and most complex of the human body, protects from the entry of pathogens and is essential for the maintenance of physiology and prevention of infections. The intestinal immune system is exposed to large numbers of antigens however is well coordinated to prevent the entry of harmful and pathogenic agents and does not react against commensal bacteria and antigens from the diet. The intestinal mucosa has a specialized defense system that is divided immune and not immune due to the constant exposure to pathogens and the ability to recognize harmful agents [47].

But when a reaction occurs against non-pathogenic particles and breaking this homeostasis inflammatory disturbances such as IBD may occur. Patients with IBD have continuously activated innate and acquired immune system with increased recruitment and retention of effector macrophages, neutrophils, T and B cell in the local inflammation where they are activated, release pro-inflammatory cytokines, and loss of tolerance to enteric commensal bacteria [14]. The higher recruitment as well as prolonged survival of effector cells caused by decreased cellular apoptosis exacerbate

the immune response and promote the accumulation of effector cells in local intestinal area, raising continuously uncontrolled inflammation [16].

1.3.4 Microbial Factors

The intestinal microbiota is a crucial factor in the development of IBD, the enteric bacteria and their production are involved in the triggering and perpetuation of the disease. The mechanism by which bacteria lead to the development of disease relates to increased site permeability and triggering of the immune response. Various microorganisms are related to the development of IBD as *Mycobacterium paratuberculosis*, *Listeria monocytogenes*, *Chlamydia trachomatis* and *Escherichia coli* [43]. The balance between beneficial bacteria and pathogenic species determines intestinal homeostasis and this balance can be manipulated by different drugs for the treatment and prevention of recurrences of IBD. The intestinal microbiome is the largest reservoir of microorganisms in the human body, having extremely useful functions, namely digestive, immune system education and repression of the growth of harmful microorganisms. The dysbiosis in IBD translates into a change in microbioma composition, diversity and quantity, microbiological changes present in both forms of IBD [47].

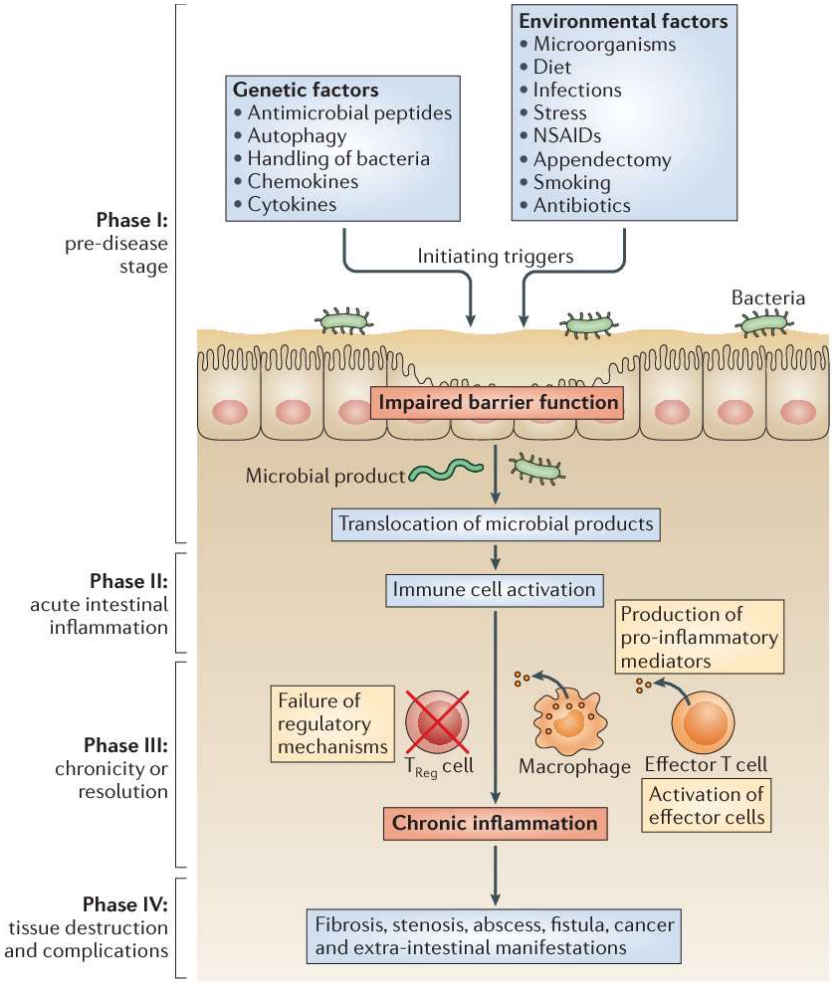


Figure 2. Conceptual framework for the pathogenesis of IBD

[adapted from Neurath, 2014]

Legend: NSAID - Non-steroidal anti-inflammatory drugs; Treg cell - Regulatory T cells.

1.4 Pathogenesis

Actually, is known that IBD is characterized by an atypical mucosal immune response and that various factors can enable this response but still not exist a defined origin to pathogenesis of IBD. However there are two different theories taking into account the awakening of the disease, the first proposes an initial dysregulation of the mucosal immune system that takes to exacerbated immunologic responses, in turn the other concept values that changes in the composition of bowel microflora associated with disturbed epithelial barrier function cause pathologic responses from the normal mucosal immune system (Figure 3) [17].

Phenotypically IBD patients have activated innate and acquired immune responses with accumulation of high levels of macrophages, neutrophils and T and B cells

recruitment in the inflamed bowel with production of pro-inflammatory cytokines and loss of tolerance to enteric commensal bacteria [16]. The enhanced recruitment as well as prolonged survival caused by decreased cellular apoptosis contribute to the maintenance of increased levels of effector cells in the inflamed intestine and consequently preservation of the inflammation and subsequent disease evolution to chronic [16]. Thus, pathogenesis of inflammatory bowel disease is not completely understood but, it is well accepted that abnormal mucosal immune response, microbial factors and epithelial cell abnormalities can facilitate this response [17].

The adaptive immune response crucial in IBD are fundamentally driven by T cells, Th0 cells become activated and differentiate into Th1 or Th2 or Th17 cells. Th1 cells are essential in the elimination of intracellular pathogens, Th2 cells are protective against parasites and mediate allergic reactions and Th17 can contribute to the clearance of extracellular bacteria [54].

Pathophysiological CD and UC are similar, however the cellular responses observed in CD and UC are different, while CD is a dominantly T helper Th1 and Th17 mediated process, UC seems to be a Th2 disorder [16].

Particularly in patients with CD it is observed a mediated cellular response dominantly Th1 and Th17. More specifically, Th1 activated T cells have the ability to produce interferon IFN-γ which in turn will activate dendritic cells and macrophages. Activated dendritic cells and macrophages produce cytokines such as interleukin (IL)-1β, IL-6, IL-12, IL-18, IL-23 and tumour necrosis factor TNF-α. Th17 polarized cells secrete IL-17 and IL-22 [55-57]. TNF-α and IL-6 are the main factor in the pathogenesis of CD, since it induces the expression of adhesion molecules in the vascular endothelium and invasion of inflammatory cells into the mucosal layer subsequently occurs [56]. Thus, it is very important controlling TNF-α and IL-6 biological action in order to ensure the regulation of this process in to control the disease [58-60].

On the other hand, it is observed in the mucosa of patients with UC a mediated response by IL-5 and IL-3 origin an atypical Th2 polarized T cell and natural killer T cells response [57,61]. Polarized T cell responses, initiate an inflammatory cascade that involves endothelial activation, chemokine production and white blood cell recruitment [55]. Triggering and perpetuation of the inflammatory response is related to deficiencies in the innate immune response caused by failures in controlling the anti-inflammatory response through IL-10, transforming growth factor b, macrophages and B cells and stromal cells and defective type I IFN production [55].

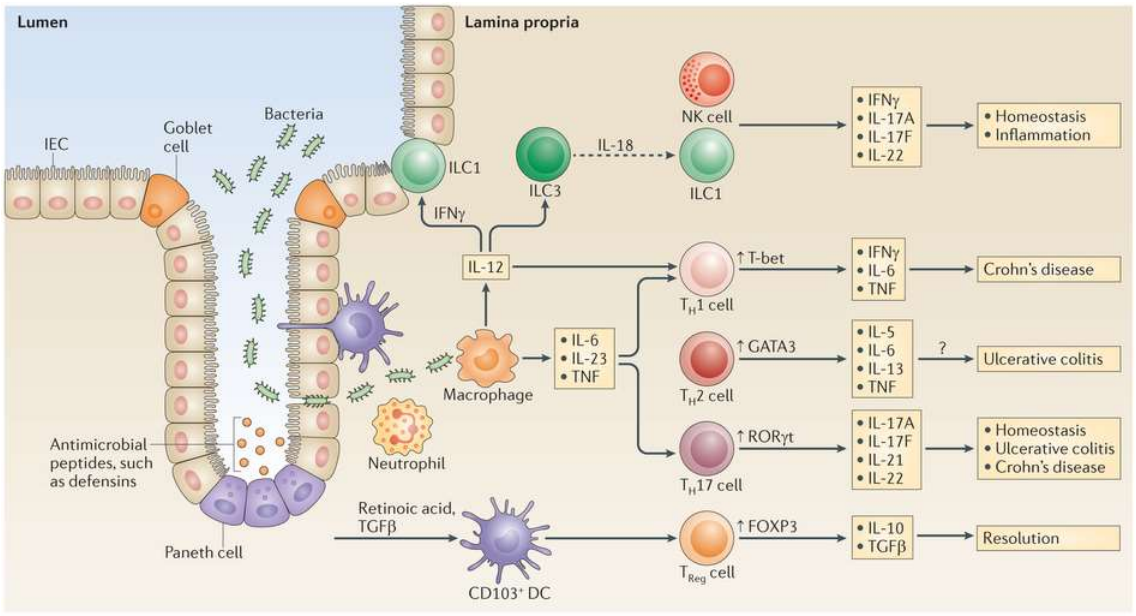


Figure 3. Cytokines in the pathogenesis of IBD [adapted from Neurath, 2014]

Legend: GATA3 – Transcription factor ; FOXP3 - Forkhead Box P3 Protein; IEC – Intestinal Epithelial Cells; IFN-γ - interferon-γ ; IL - interleukin; NK cell – Natural killer cells; ROR-related orphan receptor gamma; Th - T helper; TGF - transforming growth factor; TLR - Toll-like receptor; TNF - tumor necrosis factor; Treg - Regulatory T cells.

2. Diagnostic of Inflammatory Bowel Disease

The diagnostic of CD and UC are based on combined results obtained from different diagnostic exams available to identify colitis, along with clinical characteristics, family history of IBD and phenotypic features [31,32,41].

The major symptoms generally, depend on the intestinal affected segment and usually include diarrhea often with blood, colic abdominal pain and fecal urgency. Beyond these, other unspecific symptoms may occur like fever, loss of appetite and weight, fatigue and primary amenorrhea [15]. As well as complications like intestinal obstruction, perforation of the intestine, abscesses in the abdomen, fistulas, anal fissures and in severe cases cancer of the colon in CD and rectal bleeding, fulminant colitis and cancer of the colon in UC [31,32,41].

Laboratorial tests are enable to analyze biochemical markers to evaluate and determine the severity of colitis, like complete blood count, sedimentation rate, c-reactive protein, ferritin, serum albumin, to evaluate anemia and infection and alkaline phosphatase (ALP) determined as a marker of intestinal homeostasis once suggest damage and intestinal lesion. Urea, creatinine and alanine aminotransferase (ALT) are biochemical markers non related directly with intestine but with external organs, representative of external and consequent manifestations of the inflammation represent extraintestinal manifestations and secondary effects involved with almost every organ system and some of the most frequently involved organs like the liver and kidney [31,32]. The fecal occult blood test does the determination of fecal hemoglobin and permits the diagnosis and evaluation of various colorectal diseases, once it determines the intensity of the hemorrhagic focus in the damage colonic tissue [Hirata et al., 2007].

Stool culture is important to mislead infections caused by microorganisms and parasitic infestation, associated with cross-infection. More specifically, is used Anti-Saccharomyces cerevisiae antibodies and Perinuclear Anti-Neutrophil Cytoplasmic Antibodies which allow diagnostic precision in complex cases and distinguish CD from UC [62].

Besides these, abdominal X-ray, colonoscopy, endoscopy and biopsy even as alternative medical imaging techniques associated with clinical pattern and laboratorial tests can confirm and diagnose the presence of colitis as well as determine the extent of disease [63,64].

3. Pharmacological treatment of Inflammatory Bowel Disease

The current therapy of IBD encompasses pharmacological, nutritional and surgical treatment and aims to control the activity of the disease as well as the prevention of relapses, reduction of inflammation, resolution of complications, relief of clinical manifestations and induction of remission and its maintenance. The clinical recommendations for treatment use the signs and symptoms as markers of the activity and severity of the pathology. The severity is not directly related to the extent of intestinal involvement, but is determined by medical history, physical examination, and endoscopic and radiological studies and should be take into account as they determine the dose, dosage and formulation of the drug to be effective [34,65].

The classic clinical approach to treating IBD is called a step-up. In this approach, the treatment strategy begins with the use of less effective drugs but with better safety profile. If these drugs do not are effective in inducing the remission of the disease, it is recommended to use drugs with high efficacy but with possible more marked toxic effects. In contrast to this classic "step up" approach, proposal of a top-down approach. This approach advocates the use of biologic and immunosuppressive drugs as first-line therapy in patients in whom more complicated course of the disease, in order to avoid unnecessary use of corticosteroids, taking into account the spectrum of side effects and the high percentage of patients who are dependent or refractory to treatment with this class of drugs (Figure 4) [66].

At this point there is no knowledge of a cure, so the treatment of IBD focuses on the use of drugs that decrease the inflammatory process and induce remission of the disease. The majority of therapeutics have anti-inflammatory activity that allows the inhibition of the initial events of inflammation with the migration of inflammatory mediators, vasodilation, vascular permeability and leukocyte infiltration [4,5]. Actually, therapeutics to the treatment of IBD include a large number of drugs, like aminosalicylates, glucocorticoids, immunosuppressants, immunomodulators and biologic therapy, which have little selectivity for IBD [4,5].

In the last decade, the treatment of IBD has evolved considerably, with the appearance of several drugs directed to block specific inflammatory chain components. The search for these therapeutics that act on specific cytokines and are able to blocking the

remaining inflammatory chain, allow avoiding situations of unnecessary generalized immunosuppression and seems to be a secure bet on the future. Besides that, despite the wide range of therapeutic options mentioned above, only a part of patients achieve sustained remission with these drugs and are subject to significant long-term side effects. Allied to this it is believed in short future that the treatment of IBD pass through a combination of several cytokines capable of cover in several intracellular signaling pathways [67].

Nowadays recent advances in the pathogenesis of IBD have helped in the development of new strategies to reach new therapeutic concepts to IBD and consequently future therapies. In the future, the search for new strategies for the treatment of IBD should focus on finding specific pathways to treat the disease, contrary to what has been developed so far, which will increase the amount and quality of therapies practiced to date for IBD, progressively evolve to become broader in general options but though more targeted and individualized in patients [68].

In sum, the treatment of IBD can become complex, especially with the emergence of new therapeutic strategies, so it is essential to monitor these patients to ensure the efficacy and safety of treatment and an optimization of the adherence to therapy [69].

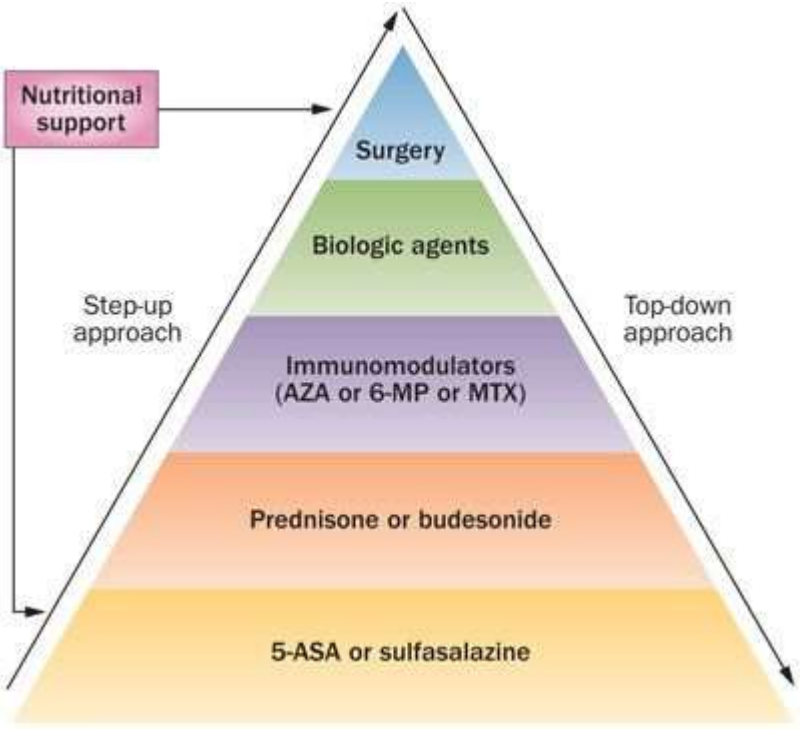


Figure 4. Therapeutic pyramid for the management of IBD

[adapted of Aloi et al., 2014].

Legend: AZA – Azathioprine; 6-MP – 6-Mercaptopurine; MTX – Methotrexate; 5-ASA – 5-Aminosalicylic acid.

3.1 Aminosalicylates

Sulfasalazine and related drugs, such as mesalamine, olsalazine and balsalazide, are the main ones used types of aminosalicylates. These medicines can suppress the symptoms when they appear and reduce the inflammation, especially in the large intestine [5].

They are derivatives of 5-amino-salicylic acid (5-ASA), used as first-line therapy UC and mild to moderate CD. These drugs have therapeutic effects at the intestinal lumen, with various oral formulations, enemas and suppositories, developed to allow better distribution of the drug [70,71].

Sulfasalazine consists of two components at the same molecule, sulfapyridine do not have substantial therapeutic effect and it believed to contribute to undesirable adverse effects and mesalamine (5- aminosalicylic acid – 5-ASA) by its time is the active therapeutic key. At the colon the two molecules combined by an azo bond are cleaved by bacterial azoreductase freeing 5-ASA and sulfapyridine [5].

Several methods of action have been implicated for these drugs, including inhibition of production of IL-1 and TNF-alpha, production of free radicals and oxidants, as well as inhibition of lipoxygenase pathway and nuclear factor kappa B however the exact mechanism of action remains unknown [73].

The vast majority of the side effects of sulfasalazine are related to the sulfapyridine molecule. The absence of this molecule in formulations of mesalazine, olsalazine and balsalazine may explain the lower incidence of adverse effects of these new drugs [70].

Mesalazine, olsalazine and balsalazine are considered to be prodrugs, with the same nitrogenous binding as sulfasalazine, however the sulfapyridine is replaced by another 5-ASA (olsalazine) or an inert compound (balsalazine). These compounds act in the same locations as the sulfasalazine, being effective in inducing maintenance of IBD remission [5].

3.2 Corticosteroids

Corticosteroids are effective in inducing remission in patients with active CD or UC, this class of drugs will suppress inflammation in its early stages, as well as late manifestations for moderate to severe relapses of IBD and in patients with no response 5-ASA oral formulations. However, are not suitable as maintenance therapy because of the severe side effects and associated with long-term use [1,73].

Corticosteroids influence the immune response of lymphocytes decrease the production of inflammatory cytokines and others inflammatory mediators as nuclear factor ƙB [74]. They act through inhibition of several inflammatory pathways like suppressing IL transcription, induction of inhibitory κB (IκB) that stabilises the NF-κB

complex, suppression of arachidonic acid metabolism and stimulation of apoptosis of lymphocytes within the lamina propria of the bowel [1].

Corticosteroids can be administered orally, as enemas, or systematically in conjunction with or without other medications. The most commonly used are prednisolone, methylprednisolone, and budesonide [73].

Corticosteroids, such as prednisolone, which is given orally, can dramatically reduce fever and diarrhea, relieve abdominal pain and tenderness, and improve appetite and sense of well-being and are the preferred steroid administered in emergency situations. However, prolonged use of corticosteroids causes side effects. Generally, high doses are taken initially to relieve inflammation and intense symptoms. The dose is then reduced, and the medicament is discontinued as soon as possible [73].

Hydrocortisone is a steroid specifically used in patients with short areas of distal proctitis and fluid retention difficulty. Budesonide, another corticosteroid, is a synthetic corticosteroid recommended as one of the first line for the treatment designed to release in the terminal ileum. Budesonide has fewer side effects than prednisolone, although it is not as quickly effective and generally does not prevent relapses after six months. Can be given orally or as an enema, because of its rapid hepatic metabolism and this drug is associated with a lower rate of side effects associated with corticosteroids [74].

3.3 Immunomodulators

Immunomodulators or immunosuppressant drugs are immunological modifiers used as maintenance therapy in patients with IBD after induction therapy with corticosteroids or surgery, patients with severe IBD, dependent or resistant to the action of corticosteroids and as treatment of first line of fistulas. These drugs are also recommended in patients with severe ulcerative colitis requiring treatment with corticosteroids or patients who need a new treatment of corticosteroids within a year. Although have side effects immunosuppressant drugs are safer than long term corticosteroid therapy and benefit from treatment seem to justify the risk. However, more studies to define the optimal duration of treatment, in order to maximize the duration of clinical remission, with a minimal risk of potentially lethal complications must be done [5].Thiopurines, azathioprine and mercaptopurine, methotrexate and cyclosporin are the most commonly immunomodulators drugs used [1].

Azathioprine and mercaptopurine are medicines that decrease the actions of the immune system. They are effective for patients with Crohn's disease who do not respond to other medications and are particularly useful for maintaining long periods of remission. They significantly improve the general clinical condition, decrease the need for corticosteroids, and often cure fistulas. However, these medications may not bring benefits before one to three months and may have potentially serious side effects [1]. Both, azathioprine and mercaptopurine pro-drugs are thiopurine analogs that function

through their active metabolite, the 6-thioguanine nucleotide (6-TGN), which leads to inhibition of ribonucleotide synthesis as well as T-cell apoptosis [37].

Methotrexate is an effective therapy in the induction and maintenance of remission in patients with IBD. Recent data demonstrate that, the methotrexate shows a considerable response rate in patients with CD or UC, intolerant or unresponsive to treatment with azathioprine or mercaptopurine, since some patients cannot tolerate and others do not respond to treatment. Treatment with methotrexate is also reserved for patients cortico-resistant or dependent.
This drug inhibits dihydrofolate reductase, blocking DNA synthesis and causing death cellular, being important in the treatment of autoimmune diseases. However, the benefits of this drug in IBD are a result of anti-inflammatory effects [1].

Ciclosporin is given in high doses injection, is used to treat IBD since 80s, as it is a medicine initially used in organ transplantation. Cyclosporine is a potent immunosuppressant drug since is an inhibitor of calcineurin, that prevents clonal expansion of T cell subsets. It acts to prevent clonal expansion of T cell subsets. At this time, cyclosporine is effective in severe UC that has failed to respond adequately to glucocorticoid therapy. This medication may help cure fistulas caused by Crohn's disease, but it cannot be used safely for a long time because of side effects such as kidney problems, infections, and seizures. Cyclosporine is effective in specific clinical settings in IBD, but the high frequency of significant adverse effects limits its use as a first-line medication [5].

3.4 Biological Therapies

Biological agents are drugs that act on specific cytokines, blocking the remaining inflammatory chain modeling the inflammatory response. In this way, the development of these biological agents will allow immune directed blockade problem, avoiding situations of unnecessary generalized immunosuppression [1]. In CD the immune responses are a characteristic Th1 cells response mediated by TNF-α, and although various cytokines are produced in the gut when inflamed, TNF- α is a major mediator of the inflammatory process. The administration of humanized monoclonal antibodies is an entirely new and potentially highly successful concept for treating IBD [5]. Currently at European level the European Medicines Agency (EMA) have several approved biological drugs like infliximab, adalimumab and golimumab to UC, anti TNF-α drugs, vedolizumab an anti-α4β7 integrin drug to CD and UC, and ustekinumab an anti IL-12 and IL-23 drug to treat CD.

Infliximab is a modulator of the immune system actions which is derived from monoclonal antibodies against tumor necrosis factor (FNT inhibitor) that binds into the form of a chimeric immunoglobulin and neutralizes TNF-α [73]. Infliximab is administered intravenously and may be used to treat moderate to severe Crohn's disease, which does not respond to other medicines, as well as to treat people with fistulas and maintain response when it is difficult to control the disease. Side effects that may occur with infliximab include worsening of an existing uncontrolled bacterial

infection, reactivation of tuberculosis or hepatitis B, and an increased risk of some cancers [1,5].
Adalimumab is a drug related to infliximab that also aims to regulate the immune system, is an anti-TNF agent that blocks TNF- α so as to counteract the inflammation [73]. Adalimumab is a fully humanized anti-TNF antibody, administered through a series of subcutaneous injections and therefore does not elicit possible reactions to the infusion of an intravenous drug, such as infliximab. Adalimumab is particularly useful for people who do not tolerate infliximab or who do not respond to it, since it is effective inducing and maintain remission in patients with CD that failure immunosuppression with other therapies [1,5].

3.5 Antibiotics

Antibiotics are useful as adjuvant therapy in severe Crohn's disease, but have limited use in ulcerative colitis. Antibiotics are often prescribed that are effective against many types of bacteria, particularly the use of antibiotics in IBD is based on the possible involvement of bacterial flora in the pathogenesis of the disease. The enteric flora is altered, and bacteria can be found in the inflamed mucosa, suggesting a possible loss of tolerance in patients with IBD. In this way, antibiotics are used in the sense of manipulating the intestinal flora, consequently, in the treatment of fistulas, abdominal abscesses, bacterial overgrowth and perianal disease [75].

Systemic side effects can limit prolonged antibiotic use. Although these symptoms generally resolve with dose reduction or cessation [37]. The most commonly used antibiotics are metronidazole, ciprofloxacin and rifaximin. [37,73,75,76].

The antibiotic metronidazole is the most frequent choice for the treatment of perianal abscesses and fistulas. Metronidazole may also help relieve the noninfectious symptoms of CD, such as diarrhea and abdominal pain. However, when given in the long term, metronidazole can damage nerves, his side effect usually subsides when the drug is discontinued, but recurrences of CD are common after discontinuation of metronidazole [1,76]. Metronidazole has been in the last decades compared to several therapies as the first line in CD and demonstrated equal efficacy in the treatment with sulfasalazine. In addition, it was effective in the treatment of patients whose treatment with sulfasalazine was not effective. The application of metronidazole in treatment of perianal disease in CD has demonstrated statistically significant effects in reducing fistula drainage and associated pain [77].

Ciprofloxacin is used, such as metronidazole, in the treatment of perianal disease, showing no significant effect on the remission rates, according to results of a pilot study by Thia et al. [77]. Nevertheless, evidence suggests a greater benefit than metronidazole in perianal CD and pouchitis [Mowat et al., 2011]. On the other hand, ciprofloxacin can lead to Clostridium difficile infection, inducing IBD relapse [76].

Rifaximin, a nonabsorbent antibiotic, is also used in the treatment of active Crohn's disease. Rifaximin proved to be significant in inducing clinical remission with minimal

side effects [37,73], however long-term treatment with high doses of rifaximin has been associated with urticarial skin side effects [76].

4. Prognosis of Inflammatory Bowel Disease

IBD is a heterogeneous condition with a highly variable clinical course, is rarely cured and is characterized by intermittent episodes of exacerbations and remissions, with some patients following a mild to moderate course while others experience early and aggressive disease progression with severe illness and frequent periods of debilitating pain. Complicated IBD can evolve to the presence of extra-intestinal manifestations and the development of colon cancer consequently with need for colon surgery, colectomy. However, with cautious clinical and surgical therapy and with maintenance therapy, many patients evolve well and successfully adapt remaining in remission for long periods of time [43].

Several clinical factors associated with complicated IBD can alter the innate pathway of disease. In Crohn's disease, young age at diagnosis, small bowel disease, upper gastrointestinal extent, stricturing or penetrating behavior, perianal disease, severe endoscopic lesions like deep ulcerations and smoking, affect the disease resolution. And in Ulcerative Colitis young age at diagnosis, male gender, extensive colitis, severe disease activity at diagnosis, high histological inflammation score, the presence of primary sclerosing cholangitis, steroid use and steroid resistance, are factors that must be taken into account with clinical course of the disease [43]. That way therapeutic strategies in IBD result from a delicate and difficult balance between benefits and risks of more gentle and aggressive therapies, taking into account the individuality of each patient and the surrounding pathophysiological situation that characterizes [1].

Disease related mortality is very low, as there is no significant risk associated with people with IBD compared to the general population [78]. However gastrointestinal cancer, including cancer of the colon and small intestine, is the leading cause of mortality associated with Crohn's disease. Patients with IBD depending on the extent and duration of the disease have increased risk of develop colorectal cancer. In patients with UC the risk of developing colon cancer is much higher when pancolitis or left-sided UC is observed contrary when UC is limited to the rectum or rectosigmoid this risk does not increase, corroborating the argument that depends on the extent and duration of the disease the risk of developing cancer as well as the treatment used in the life time of the disease [33].

Chapter 2 - Animal Models of Inflammatory Bowel Disease

1. Animal models of Inflammatory Bowel Disease

Experimental models of inflammatory bowel disease supply considerable information on the pathogenesis of this illness, represent an important tool in testing new strategies of treatment and playing a pivotal role in the development of novel therapeutic drugs once they are capable of characterizing physiologic interactions when our understanding of such processes is insufficient to permit replacement of in vitro systems [79]. Due to the complexity of the pathophysiology of IBD, the experimental animal models used to study these diseases do not accurately represent their characteristics, however one IBD patient does not entirely resemble another, hereupon, the diversity of responses we observe in animal models is no different than what we observe in humans making these animal models useful and valid tools that allow the investigation of factors related to pathology and also new strategy therapies [80]. Currently used therapy aim to induce and maintain the patient in remission and ameliorate the disease´s secondary effects, rather than modifying or reversing the underlying pathogenic mechanism, this treatment difficulties lead to the need for further preclinical studies with the aim of testing new pharmacological approaches, hence the need and pertinence of validating animal models of disease that mimic faithfully the disease in human (Table 6) [4,5].

There are several types of animal models of IBD, however, experimental models used actually are classified in four groups, based on how the disease is formed. Current exist spontaneous colitis animal models, as they develop the disease naturally in their environment, however, is not a model regularly used due to randomness of disease development and difficulties in find these animals, as well as breeding animals to develop IBD with this model. The induced colitis by specific immunological or chemical agents models normally induce physically an immune response and epithelial damage according to induced agent used. A third group, the genetically modified animal models by gene knockout, knockin or transgenic methods allow uncover the genetic factor and subsequently the candidate gene underlying the disease but are not able to preclinical efficacy testing once this model uses a genetic engineered population were only one gene is modified in an restricted controlled environment, impossible resemble IBD in humans. The last model includes adoptative transfer models, this immunological model induces experimental inflammation through transferring specific T cells into an immune compromised individual, however due to the extremely complex protocol many factors can lead to bias in results, making it a difficult model to mimic IBD [7].

Ideally, a disease model should closely parallel the human disease in clinical manifestations, pathophysiology, and response to existing therapeutic elements. However so many animal models are now available and various features like species, strains, subtrains, the microenvironment in which animals live and the mediators involved in each of these models must be taking into consideration making it difficult to the researcher to choose the most appropriate model to use for preclinical testing [81,82].

Table 6. Advantages and limitations of using animal models

[adapted from Vandamme, 2015]

Advantages	Limitations
Availability of different models;	Difference between species;
Direct information;	Variations induced by techniques;
Possibility to create new models;	Differences in genetic regulation;
Greater analytical potential;	Anatomical differences;
Controlled modification of variables.	Various pathophysiological mechanisms;
	Different responses to drugs.

In this view, one of the most commonly used and studied in greatest detail animal model is colitis induced by administration of chemical substances, which are toxic to colonic cells, generate intense inflammatory response, recruitment of inflammatory cells, representing some of the characteristics observed in human disease. Chemically induced colitis is a simple model to be directed, whose disease onset, duration and severity can be manipulated [7]. These models require the co-administration of a substance that temporally disrupts the mucosal integrity and allow the colitogenic components to access the mucosal immune system (Table 7) [6]. There is interest in the use and study of more than one animal model since differences between models may reflect the different subgroups of patients with IBD, given this, the most commonly used are TNBS induced colitis model which promotes a Th1 response, resembling CD and DSS induced colitis model that promotes a Th2 response, resembling UC. In practice, this pattern of T-cell differentiation is associated with distinct functional activities: Th1 T cells are the key players in delayed type hypersensitivity reactions, whereas Th2 T cells are potent inducers of antibody mediated immunologic reactions [6,83]. Dextran sulphate sodium (DSS) induced colitis and TNBS induced colitis models are the most widely used to induce IBD since they symptomatically, morphologically and histopathologically resemble human IBD and allow the development and test of novel therapeutic strategies [6,84,85].

Table 7. Animal chemically induced models of IBD [adapted from Murthy, 2006]

Model	Species	Method of induction	Time course	Disease location	Type of colitis
TNBS	Rats, mice and rabbits	TNBS enema (20-30mg in 30-50% EtOH)	3 days – 8 weeks	Small intestine or colon	Acute and chronic
DSS	Hamsters, mice and rats	2 - 10% DSS feeding	5 days – 15 weeks/	Colon	Acute and chronic
Acetic acid	Rats	1 – 10% acetic acid enema	1 day – 3 weeks	Colon	Acute
Carrageenan	Rats, guinea, pigs and rabbits	Variable oral dosing	1 – 4 weeks	Cecum and colon	Acute and chronic
Indomethacin	Rats	Oral or SC once or twice	< 1 - 8 days	Small intestine	Acute
Oxazalone	Mice and rats	Intracolonic	Rapid	Colon	Acute

Legend: DSS - Dextran Sulfate Sodium, TNBS - Trinitrobenzene Sulfonic Acid

1.1 Dextran sulfate sodium-induced Colitis

DSS is actually one of the most regularly used colitis inducer to trigger bowel inflammation in animal models, largely due to is ease of use and brief obtained results and that morphologically and symptomatically resembles UC in humans [7,86].

To cause inflammation in rats or mice DSS protocol uses DSS added to drinking water, then acute or chronic colitis model experiments can be conducted only by altering the concentrations of the administered substance as well as the number of cycles of supply of the chemical agent. The severity of colitis caused by DSS depends on dose, duration of administration and animal strain [86-88], as well as, the manufacturer and molecular weight of DSS, gender and animals raising environment like germ-free or specific pathogen free environments [89,90].

DSS seems to induce colonic damage through a chemically process where the gut mucosa barrier integrity is injured, allowing luminal antigens access to the lamina propria and the pro-inflammatory cells [90]. Furthermore, the bacteria present in gut are able to dissociate sulfate from DSS, leaving free in the bowel a sulfate molecule which

helps as substrate to produce hydrogen sulfide. Hydrogen sulfide induce a toxic effect on epithelium since it might expressively affect cellular metabolism. On the other hand, DSS probable promotes variations in luminal bacterial ecosystem and the activation of monocytes, macrophages and mast cells [7,91]. DSS increases the production of all pro-inflammatory cytokines in colon, particularly TNF-α levels and promotes chronic pathological changes which involve changes in the morphology of crypts with changes in Th1/Th2 cytokine profile and central memory T cells. Besides that, have been shown that DSS induced significant macrophage infiltration into the epithelium of the colon [92].

The DSS colitis model is very popular in IBD research due to its rapidity, simplicity, reproducibility and controllability. The addition of DSS to drinking water, modifying the concentration of DSS and the frequency of administration permit obtain a very reproducible acute or chronic and relapsing model of intestinal colonic inflammation, as well as a useful model for a better understanding of the innate immune mechanisms of UC [7]. DSS is usually administered in a dose range of 2-10% for 5-10 days to induce acute inflammation following a single continuous exposure. By prolonging DSS administration, acute colitis may be extrapolated to chronic colitis by repeated exposure administered in three to five cycles interrupted with recovery periods [6,84,85]. However, DSS model is very expensive among other disadvantages like variations in disease severity could occur due to small molecular weight DSS impurities in the DSS preparation. Besides, the disease is characterized by progressive crypt dropout, suggesting a direct effect of DSS on the epithelial cells as opposed to lamina propria cells as suggested in human IBD [91].

1.2 Trinitrobenzene sulfonic acid-induced Colitis

TNBS model is a commonly used model of IBD to study the pathogenesis of colitis as well as in preclinical studies, in 1989 was described by the first time by Morris and since then have become a popular and important tool mimic the pattern of inflammation in human IBD. TNBS is capable of reproduce CD in humans once promotes a Th1 immune response with characteristic chronic transmural colitis and consequently severe diarrhea, weight loss and rectal prolapse. TNBS model is an easy induced, rapid, reliable, robust and a highly reproducible animal model of intestinal inflammation. The induction of the disease occurs quickly and appears 4 to 7 days after intrarectal administration of the TNBS hapten reagent, gradually progressing into chronic pattern during at most approximately about 8 weeks [83,93,94].

Inflammation caused by TNBS histologically is characterized by focal ulcers, crypt distortion, depletion of goblet cells, presence of granulomas, thickening of the intestinal wall, edema, infiltration of inflammatory cells and necrosis [7,8].

Protocols of the chronic TNBS-induced colitis model are not standardized concerning the dose of TNBS, the depth of TNBS administration, the animal strain, and the time point for model evaluation [7].

The experimental model with TNBS consists in the induction of intestinal inflammation by a chemical process in which colitis is induced by rectal instillation of 20-80mg of 2,4,6-trinitrobenzenesulfonic acid in an ethanol solution of 30-50% , ethanol will act to break the membrane, whereas TNBS acid would act with a hapten which associated with substances with high molecular weight such as tissue proteins, would be able to trigger an immune response. This model is based on increased permeability of the membrane that occurs in IBD, which facilitates the entry of a luminal antigen that is not adequately eliminated by the immune system, the haptenization [82,95]. Ethanol, by his side, permeabilizes the epithelial layer that separates the luminal contents of the colon from the cells of the mucosal immune system, allowing the penetration of TNBS in the bowel wall (Figure 7) [82,96].

TNBS induces acute and chronic form of colitis dependent of dose and frequency of administration, reacting with some amino acid groups on intestinal mucosa and bacterial proteins of the colon and rendering them immunogenic. TNBS haptenates autologous colonic proteins with a trinitrophenyl moiety and induces an IL-12 mediated Th1 T cell transmural colitis, which resembles human IBD, both on a histologic and immunologic level. TNBS associated with ethanol promotes the development of severe, transmural, granulomatous inflammation in the distal colon [82,95].

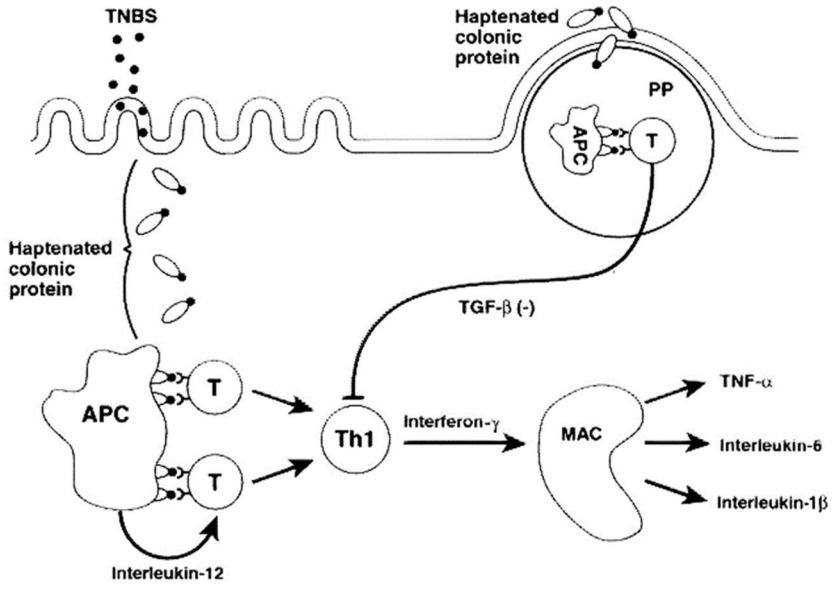


Figure 5. Mechanism of colitis induction and tolerance in the TNBS-induced colitis model [adapted of Strober et al., 1998]

Legend: TNBS - Trinitrobenzene sulfonic acid; APC – Antigen presenting cells; MAC - Membrane attack complex; TNF-α - Tumour necrosis factor α.

With this model induction occurs increase enzymatic activity of myeloperoxidase and alkaline phosphatase and increased expression of pro-inflammatory cytokines such as TNF-α and interleukins, as well as changes in the antioxidant systems of the intestinal mucosa. The presence and recognition of TNBS promotes IL-12 secretion and induces the response of Th1 cells, which produce INF-γ, this acts on macrophages inducing the production of pro-inflammatory cytokines such as TNF- α, IL-1 and IL- 6, triggering the

inflammatory process. In addition to these other inflammatory mediators are involved as prostagrandins E2, thromboxane B2, leukotriene B4 and C4 and other interleukins [7,8,97].

The main advantages of this model include a simple and low-cost protocol and reproducible colonic damage, short experiment duration, enduring damage accompanied by inflammatory cell infiltration and ulcers. On the other hand, the TNBS colitis model has some disadvantages, such as the absence of spontaneous relapse that is the hallmark of IBD and model reproducibility depends on the TNBS dose [97].

Thus, TNBS chemically induced model is a useful and important experimental model to mimic IBD, as it allows the study of the initial events of inflammation and the subsequent analysis of the intestinal mucosal immune response triggered by a specific antigen and consequent pathogenesis and new metabolic pathways of the disease, making it possible to evaluate various mediators involved in the inflammatory response and new compounds to treat UC or CD to achieve new pharmacological approaches to the treatment of the disease [7].

Chapter 3 – Materials and Methods

Chemicals/Materials

2,4,6-Trinitrobenzene sulfonic acid (TNBS 5%) and sodium chloride (NaCL) were purchased from Sigma Chemical Co. Ketamine (Imalgene® 1000) was purchased from Merial. Xilazine (Rompun® 2%) was purchased from Bayer. ADVIA® kit was purchased from Siemens Healthcare Diagnostics. ELISA assay kits for TNF-α and IL-10 measurements were obtained from Hycult Biotechnology.

Animals

The mice female CD-1 were obtained from Charles River – Barcelona, Spain, with weight of 25-40g and 6-10 weeks of age. The animals were kept in the Molecular Medicine Institute in Faculty of Medicine of the University of Lisbon biotereum under controlled conditions of access to water and food (ad libitum), at a temperature between 18-23ºC and humidity of 40-60% in standard polypropylene cages. Animal care was in strict accordance with internationally accepted principles for laboratory animal use and care, Directive 2010/63/EU. The experiment was approved by the institutional animal ethics committee of Faculty of Pharmacy, University of Lisbon.

Induction of Experimental Colitis

In this study was used a chronic chemical model of colitis induction with multiple administrations. The animals were submitted to a briefly fasting period of 24h. In the induction day (day 0) the mice were anesthetized with 40µl ketamine 100mg / kg + xylazine 10mg / kg by intraperitoneal injection (IP). Thereafter, the rectal administration of 100µl TNBS solution was performed by introducing a catheter up to 4cm into the mice colon. To prevent colonic reflux the mice were post-maintained in Trendelenburg position [7]. The procedure was repeated weekly, at day 7 of each week, during a period of 6 weeks in order to obtain a chronic model of colitis induced by TNBS. Every week, on days 7, 14, 21, 28, 35 and 42, depending on the experimental group respectively, blood samples of mice were collected by cardiac puncture, under anesthesia, immediately before euthanasia. Mice were sacrificed by cervical dislocation and necropsied. The abdomen was opened with a middle incision and the colons were removed, released from enclosed tissues, washed with phosphate-buffered saline. Colon length was also measured as a marker of tissue integrity, determined using a measuring scale and analyzed for intestinal damage, taking into account macroscopic, histopathological and biochemical parameters of the inflammatory process. Macroscopic score evaluation of the colon was based on described by Morris et al 1989 [8], Criteria for scoring of gross morphologic damage (Table 8), together with the evaluation of presence of diarrhea and adhesion in colon.

Table 8. Criteria for Scoring of Gross Morphologic Damage

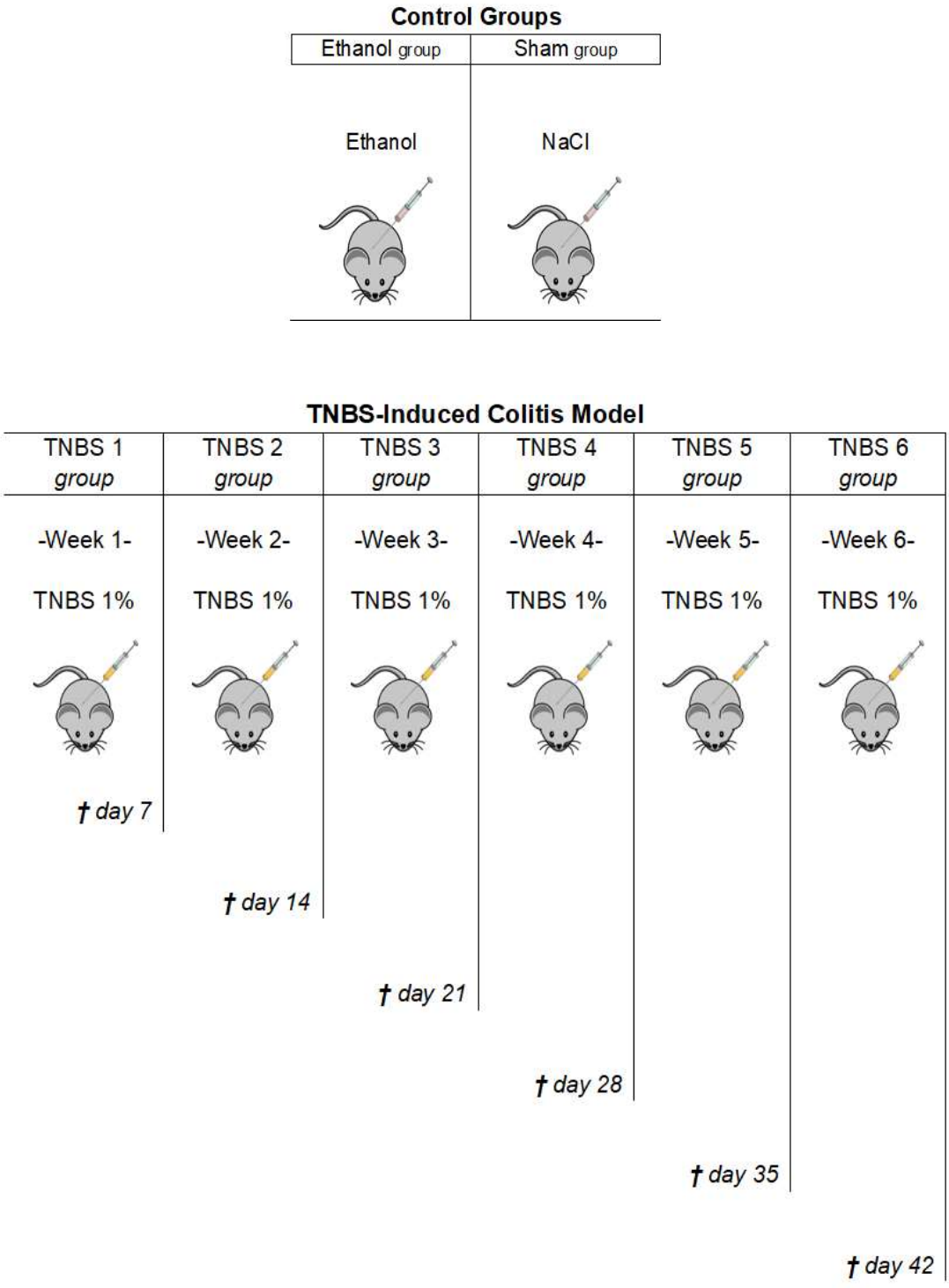
[adapted from Morris et al. 1989]

Score	Gross morphology
0	No damage.
1	Localized hyperemia, but no ulcers.
2	Linear ulcers with no significant inflammation.
3	Linear ulcer with inflammation at one site.
4	Two or more sites of ulceration and/or inflammation.
5	Two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending >1 cm along the length of the colon.

Experimental Groups

An experiment was performed on the development of the procedure where mice were randomized into 8 groups, accordingly to the main objective of this study, namely the development of a TNBS induced chronic colitis model (Table 8). Experimental groups were categorized as TNBS 1 group (n=15), TNBS 2 group (n=15), TNBS 3 group (n=15), TNBS 4 group (n=15), TNBS 5 group (n=15) and TNBS 6 group (n=15), received all 100µl intrarectal 1% TNBS in 50% ethanol weekly, depending on the number of administrations; ethanol group (n=15) received 100µl intrarectal 50% ethanol (TNBS vehicle) and sham group (n=8) received 100µl intrarectal saline solution. All groups were maintained under the same conditions. The animals in the control groups were placed in cages separated from the cages of the animals that would undergo colitis induction. The ethanol and sham group were used as reference to compare with other experimental groups (Table 9).

Table 9. Scheme of study design of the experimental groups.



Legend: † - Euthanized on

Monitoring of Clinical Signs

During the experimental development the animals were observed daily and monitored clinical signs throughout the evaluation of different parameters such as body weight, morbidity, stool consistency and anus appearance.

Biochemical Markers

Before the mice be killed, were anesthetized and blood samples collected by cardiac puncture. Serum collected from blood samples were separated by centrifugation at 3600rpm for 15 minutes and analyzed by an automated clinical chemistry analyzer (ADVIA 2400). The biochemical markers such as alkaline phosphatase (ALP), urea, creatinine and alanine aminotransferase (ALT) were evaluated spectrophotometrically, and a quantitative method by immunoturbidimetry (Kroma Systems) was used to evaluate fecal hemoglobin, as an index of hemorrhagic focus. Alkaline phosphatase (ALP) was determined as a marker of intestinal homeostasis, urea was determined as a marker of renal function, creatinine as a marker of renal function and alanine aminotransferase (ALT) was determined as a marker of hepatic function.

Measurement of Cytokines

The pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10 were measured in the colon according to the manufacturer's recommended protocol, and expressed as pg/ml. To cytokine dosing, colon samples were weighed and homogenized (Ultraturrax T25, 13.500rev/min, twice for 30s) in phosphate buffer and centrifuged at 15.000rpm for 15min at 4ºC. The samples of supernatant were kept at -20o C for further enzymatic assay. The reaction to determine the cytokine levels was stopped and the plates were measured in a spectrophotometer at 450nm (ELISA kit Quantikine, Hycult Biotechnology).

Hystopatological Analysis

The histopathological analysis of bowel tissues from all mice groups was done by independent histopathologists blinded to the treatment groups from Institute of Molecular Medicine (IMM). The colon samples of the animals were removed shortly after euthanasia and fixed in 10% phosphate-buffered formalin and processed for paraffin embedding. After, the tissues were sectioned at 5μm and stained with hematoxylin and eosin. Longitudinal sections of distal colon were evaluated based on adapted criteria of Corazza and colleagues [9] and Seamons and colleagues [10].

Microscopic Assessment of Colitis Severity

The sections were examined and scored according to the presence of inflammation (0-4 increasing severity) with some parameters, namely: (1) presence of tissue loss/necrosis, (2) severity of mucosal epithelial lesion, (3) inflammation, (4) extent 1 - the percentage of intestine affected in any manner and (5) extent 2 - the percentage of intestine affected by the most severe lesion. The colitis severity was calculated by

summing the individual lesions and the extent scores, promoting a final colitis score (max score=20)(Table 10).

Table 10. Scoring system of histopathologic evaluation of TNBS induced colitis.

Points	Tissue Loss	Epithelial Lesion	Inflammation	Extent
0	None	None	None	None
1	Mucosa ≤ 50%	Mild mucous cell depletion	Mild - mucosa only	≤5%
2	Mucosa ≥ 50%	Moderate mucous cell depletion	Moderate - mural	6-30%
3	Mural	Severe with aberhant crypts	Transmural	31-60%
4	Transmural	Severe with cryp loss	Extension to the mesentery	≥61%

Statistical Analysis

All the results were statistically analyzed with a p-value of less than 0.05 considered significant. All results were expressed as mean ± SEM of N observations, where n represents the number of animals studied. Data analysis was performed by using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). The results were analyzed by one-way ANOVA to determine statistical significance between the experimental and control groups followed by Tukey's post hoc test for multiple comparisons or chi-square test depending on the variables under study.

Chapter 4 – Results

The induction of the acute disease occurs 4 to 7 days after intrarectal administration of the TNBS hapten reagent, gradually progressing into chronic [11,8,12]. Since the protocols of the TNBS-induced colitis model are not standardized the experimental protocol was performed, with six independent groups of TNBS-induced colitis and developed and monitored under the same specific conditions. Mice were sacrificed 7 days after each instillation, during a period of 6 weeks. Thus, to identify the chronic pattern associated with IBD with the induction method used on this study.

Monitoring of Clinical Signs

The mice were observed daily during the complete experimental protocol taking into account parameters like morbidity, body weight, stool consistency and anus appearance. On week 2 until week 4 the generality of mice presented alterations of intestinal motility characterized by soft stools, slight edema of the anus and moderate morbidity. Apparently, after week 4, the mice presented adapted to TNBS and an apparently recover, revealing less exacerbated clinical signs and consequently less morbidity. With reference to ethanol and sham groups, taking into account the same clinical signs during, all experimental period, were not identified any alteration, according to control groups. Concerning body weight, all TNBS groups demonstrated a very similar curve in the register of body weight each week (Figure 8). Until week 4 was observed a progressive increase in body weight common to all groups. In turn, on week 3 a slight decrease in mice body weight of the groups was observed. Ending the TNBS 6 group, mice had more 13.32 ± 2.7% of its initial weight. The ethanol and sham groups presented a considerable increase in body weight. At the end of experimental period, the ethanol and sham groups gained 12.74 ± 2.3% and 13.08 ± 2.4% of its initial weight, respectively.

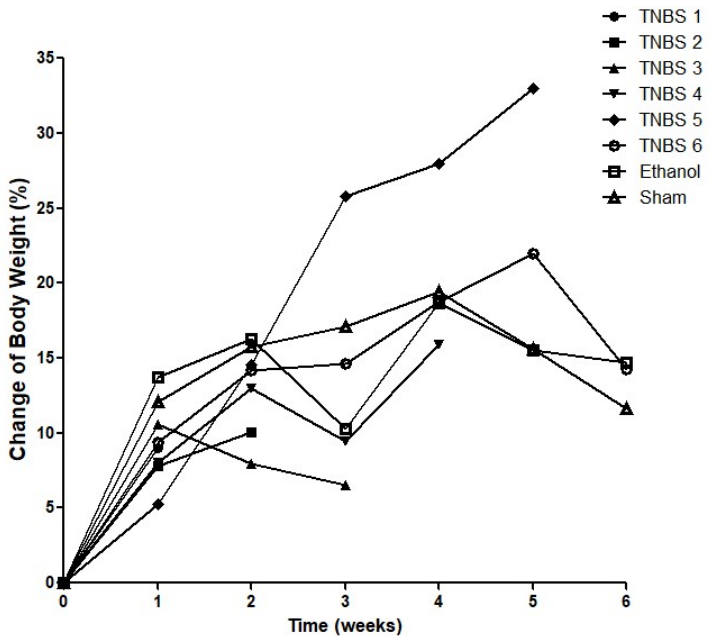


Figure 6. Change of body weight during the development of TNBS-induced colitis

The colons were observed macroscopically and scored to gross morphology according to the Morris method. The maximal damage in the colon where observed with two instillations, at group TNBS 2, the score mean observed was 2.67 ± 0.67 ($p<0.0001$) correspondent with the classification, linear ulcer with inflammation at one site. By comparison at week 4 onwards the evaluation score decreased to 0.43 ± 0.20 ($p<0.0001$) correspondent with localized hyperemia, but no ulcers. The control groups, ethanol and sham present a colon classification of no damage with a score of 0 to both groups (Figure 9).

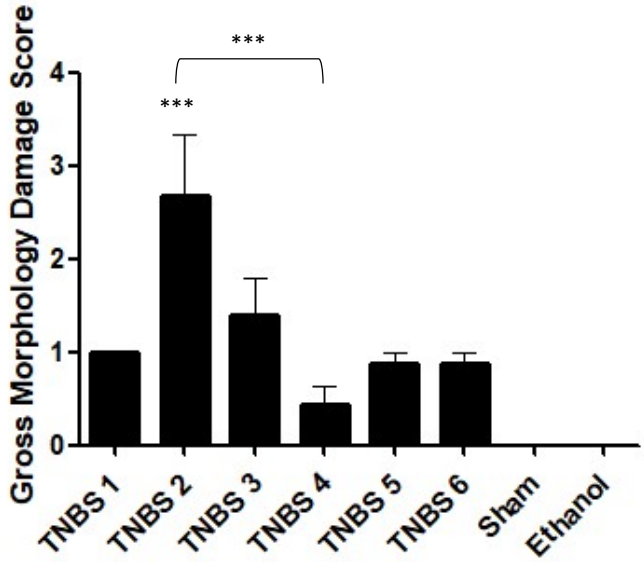


Figure 7. Gross morphology damage score during the development of TNBS-induced colitis.

Legend: One-way ANOVA and Tukey's post hoc test; *** $p<0.0001$ compared with sham group or between groups.

Survival curves indicate the probability that the product will survive until a certain period of time. The survival curve designed by our study group demonstrates along the experimental procedure a high-pitched drop in survival at the first two weeks. In the first week survival decreased to approximately 62%, and in the second week a new decrease occurs to values of 50%. After week 2 onwards the mice survival maintains the same values until the end of the experimental protocol indicate stability and adaptability of animals to disease (Figure 10).

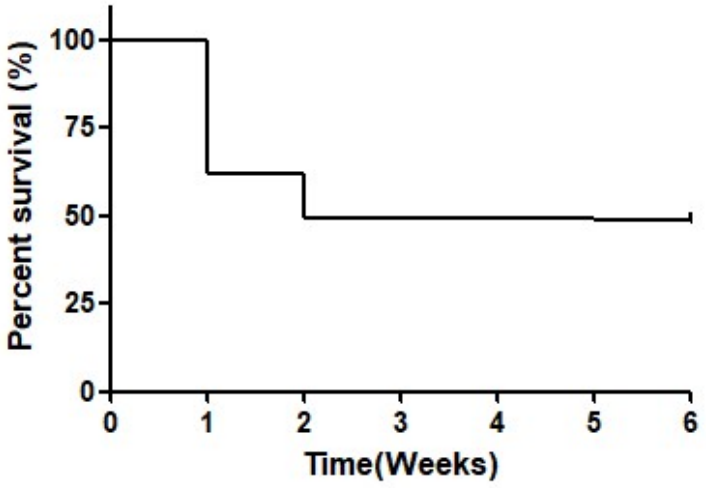


Figure 8. Effect of TNBS-induced colitis on survival in the IBD.

Biochemical Markers

The colon length was determined at the end of the treatment period, using a measuring scale (Figure 11). The influence of TNBS induced colitis in the colon length comparing with control groups was observed, however with no statistical significance. The TNBS groups did not display significant changes compared to the sham group. More specifically, the TNBS 4 group presented around 8.5 ± 0.21 cm of colon and, contrary to what was observed in the initial week, TNBS 1 has 9.9 ± 0.55 cm, values that remained more or less constant over the course of the experience, TNBS 6, with 8.7 ± 0.30 cm. On group TNBS 4, the colon length of TNBS group was considerably lower than control groups, as ethanol and sham groups, which presented 9.2 ± 0.3 cm and 9.5 ± 0.5 cm of colon, respectively.

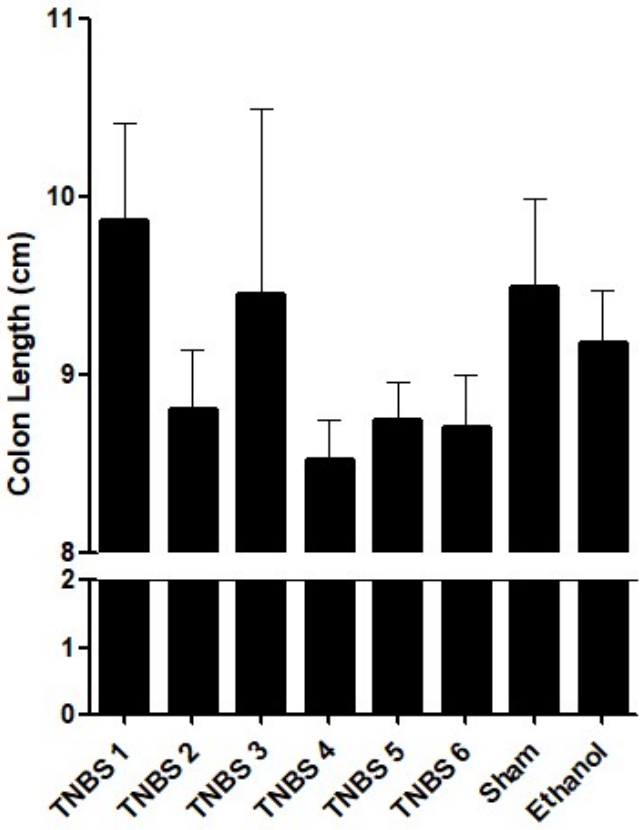


Figure 9. Effect of TNBS-induced colitis on colon length in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test.

The fecal hemoglobin was evaluated as an index of hemorrhagic focus in the damage colonic tissue (Figure 12). On week 2, the mice with colitis had 2.6 ± 0.3 µmol Hg/g feces and, in the week 6 of administration of TNBS, the fecal hemoglobin considerably increased to 7.5 ± 0.5 µmol Hg/g feces ($p<0.0001$). Thus comparing TNBS 4 fecal hemoglobin values with TNBS week 6, no statistical significance was observed. By his side the comparation between TNBS and sham group demonstrate an augmented level of fecal hemoglobin in the end of the experiment ($p<0.0001$). The control groups presented very low concentrations of fecal hemoglobin with 2.1 ± 0.24 µmol Hg/g feces on ethanol group and 1.6 ± 0.12 µmol Hg/g feces on sham group.

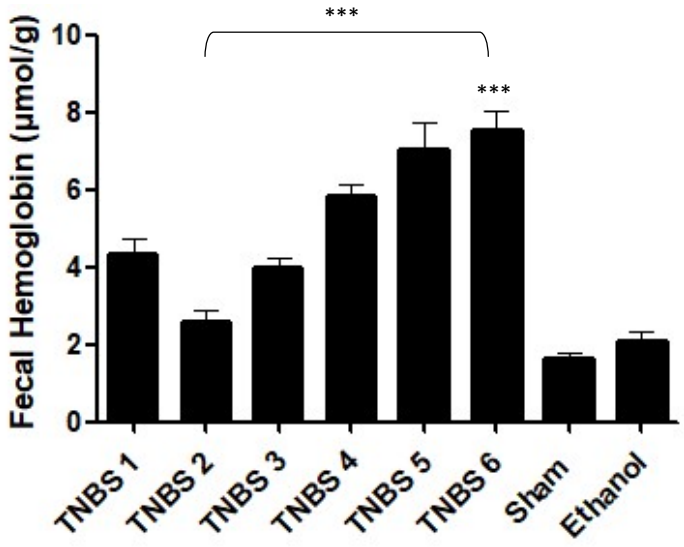


Figure 10. Effect of TNBS-induced colitis on fecal hemoglobin in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.0001 compared with sham group or between groups.

The ALP dosing is associated to the presence of intestinal lesion hereupon it was evaluated his concentration on blood (Figure 13). At TNBS 2 a decreased value of ALP was dosing in serum comparing with sham group, 21.67 ± 2.7IU/L, however with no statistical significance. The ALP levels observed were in the majority higher than those observed by the control groups, with a maximum level at week 5 of 58.5 ± 2.18 IU/L, compared with sham group (p<0.0001). Nevertheless, according to the different parameters analyzed the maintenance of the values after week 4, 39.67 ± 2.45 IU/L, until the end of the experiment, TNBS 6, 44.38 ± 2.3IU/L, with no statistical significance. Ethanol and sham groups presented concentrations of ALP with around 33.17 ± 1.5 IU/L and 30.5 ± 0.88 IU/L, respectively.

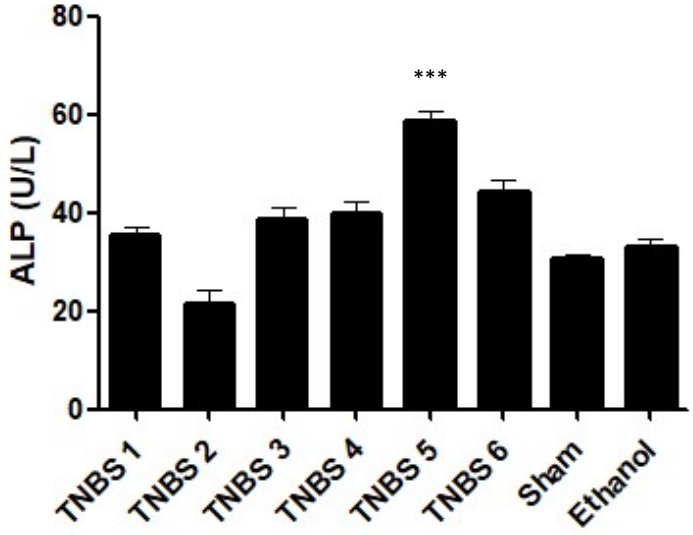


Figure 11. Effect of TNBS-induced colitis on serum total ALP concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.0001 compared with sham group.

Urea and creatinine are biochemical markers nonrelated directly with intestine allowing the assessment of renal damage based on urea and creatinine concentrations (Figure 14 and 15). Regarding blood urea levels starting to verify a similar dosing result over the entire trial period. The TNBS 1 and sham groups presented a urea concentration of 83.0 ± 1.55 mg/dl and 81.6 ± 0.86 mg/dl, respectively. Comparing TNBS 6 with sham group (77.4 ± 1.86 vs 81.6 ± 0.86 mg/dl), urea maintained levels of the same order of magnitude, with no statistical significance observed. By his side creatinine had a similar values pattern, when compared with the ethanol group, the TNBS group 1 (0.54 ± 0.01 mg/dl) had a significantly higher creatinine concentration ($p<0.01$), comparing with ethanol group, 0.41 ± 0.03 mg/dl. By his side TNBS 1 comparing with TNBS 3 shows decreased values with statistical significance ($p<0.01$). TNBS 4 with ethanol group demonstrate statistical significant values, with high levels on week four ($p<0.001$). TNBS 6 compared with sham group (0.51 ± 0.02 vs 0.43 ± 0.02 mg/dl), reveals no statistical significance. The ethanol group presented 77.17 ± 2.20 mg/dl of urea and 0.41 ± 0.03 mg/dl of creatinine. There are no statistically significant differences in urea and creatinine levels comparing to the control groups.

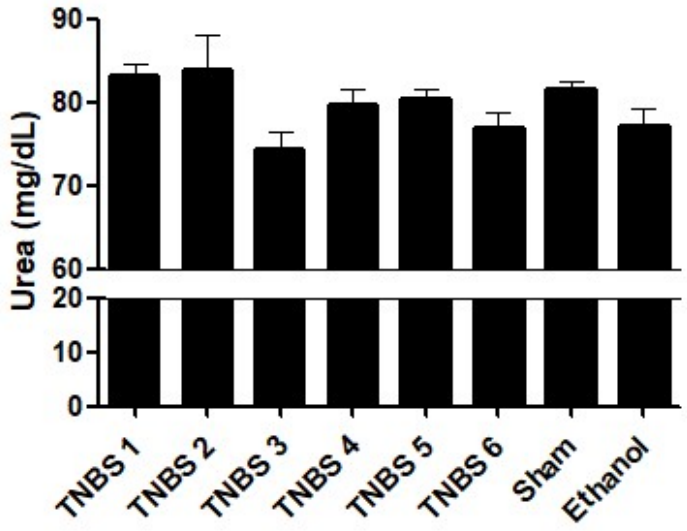


Figure 12. Effect of TNBS-induced colitis on serum urea concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test.

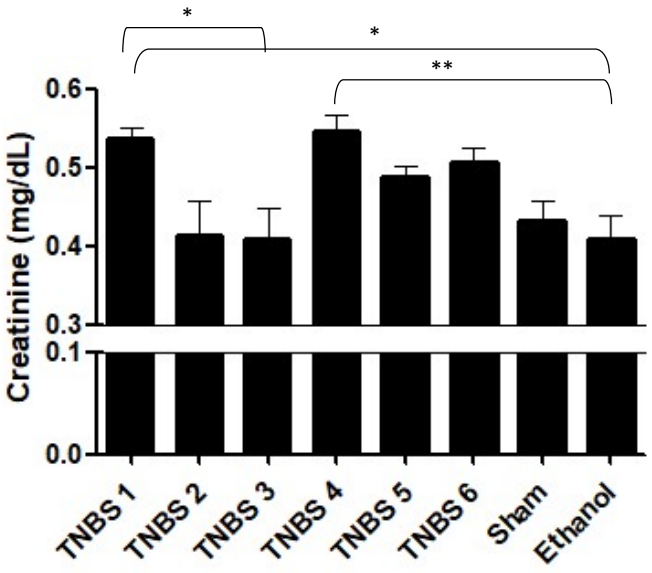


Figure 13. Effect of TNBS-induced colitis on serum creatinine concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; * p<0.01 compared with ethanol group or between groups; p<0.001 compared with ethanol group.

ALT concentration in serum was evaluated as a hepatic marker of liver homeostasis (Figure 16). TNBS group 1 ALT levels were 28.14 ± 1.96 IU/L comparing with sham group (p<0.01). The ALT concentration starts to increase significantly in the TNBS week 4 group compared with the sham group (35.0 ± 1.6 vs 19.3 ± 0.54 IU/L, p<0.0001). On week 6, the ALT maintained approximately the values 35.75 ± 1.5 IU/L compared to TNBS day 4 group, but yet for higher values than sham group (p<0.0001). The ALT concentration on ethanol group (24.67 ± 1.69 IU/L) was higher than the sham group (19.30 ± 0.54 IU/L) although without statistically significant differences.

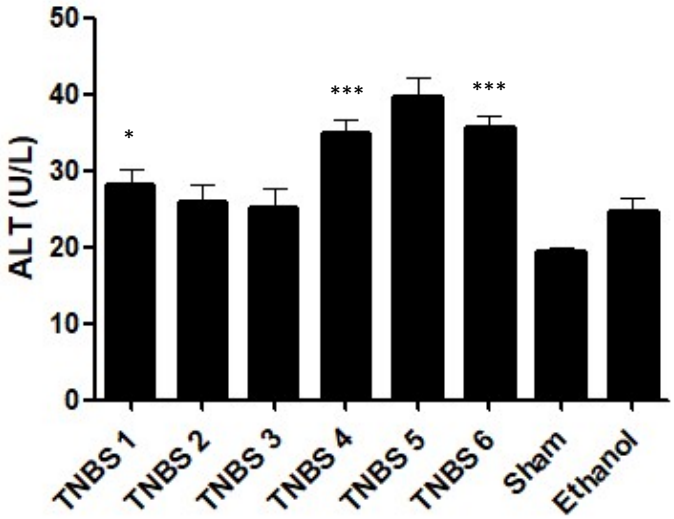


Figure 14. Effect of TNBS-induced colitis on serum ALT concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with sham group or between groups.

Measurement of Cytokines

The TNBS-induced colitis showed a significant production of pro-inflammatory cytokines, as TNF-α comparing to sham and ethanol groups (Figure 17). All TNBS groups presented higher levels of pro-inflammatory cytokines than sham group. The TNBS 1 group has higher values statistically significant, when compared with group TNBS 2, (79.59 ± 5.89 vs 51.5 ± 2.9 pg/mL, p<0.001). The highest level of TNF-α was registered at TNBS 3 administration, 87.58 ± 13.13 pg/ml, compared with week 6, 72.70 ± 3.6 pg/ml, without statistical significance, indicating a maintenance in the values along with time. The TNBS 4 was also compared with sham group, since statistically significant values were observed demonstrate the augmented pro-inflammatory cytokines levels in this model (71.34 ± 3.28 vs 39.69 ± 2.4 pg/mL, p<0.001). Even, the ethanol and sham groups had very similar concentrations of pro-inflammatory cytokines, without statistically significant differences. The ethanol group presented 39.80 ± 1.5 pg/ml of TNF-α and the sham group presented 39.69 ± 2.4 pg/ml.

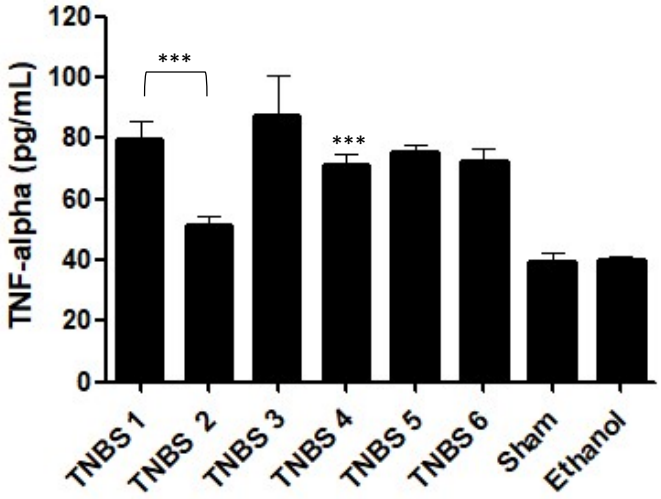


Figure 15. Effect of TNBS-induced colitis on TNF-α concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.001 compared with sham group or between groups.

The TNBS-induced colitis showed a significant production of anti-inflammatory cytokines, IL-10, (Figure 18). Contrary to expected increased IL-10 values are observed simultaneously with the increase of pro-inflammatory cytokines such as TNF-α. Usually the IL-10 decrease when the TNF-α concentrations increase under inflammatory conditions. The TNBS 1 group as 53.39 ± 3.17 pg/ml when compared with sham group, 33.99 ± 1.7 pg/ml, p<0.01. Comparing TNBS 3 with TNBS 6 an increase is observed congruent with the evolution of pro-inflammatory cytokines along with the inflammation (44.56 ± 3.73 vs 68.21 ± 1.76 pg/mL, p<0.0001). At week 6 the IL-10 values were 68.21 ± 1.76 pg/ml without statistical significance when compared with TNBS 4 group, 57.34 ± 3.81 pg/ml, demonstrate a constant level of IL-10 along with the experimental study. The control groups, as ethanol and sham groups, had similar results with 43.73 ± 3.7 pg/ml and 33.99 ± 1.7 pg/ml of IL-10, respectively.

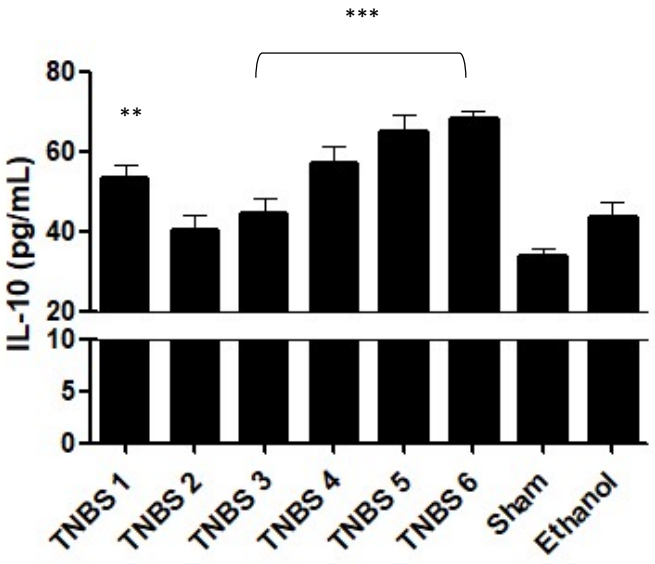


Figure 16. Effect of TNBS-induced colitis on IL-10 concentration in the IBD.

Legend: One-way ANOVA and Tukey's post hoc test; *** p<0.0001 compared with sham group or between groups, **p<0.001 compared with sham group.

Hystopatological Analysis

The data regarding histopathological analysis performed in the studied colons were not obtained during the period of development of this dissertation.

Chapter 5 – Discussion

IBD is a chronic inflammatory disorder of the epithelium of the intestinal tract caused by multiple factors and for which therapeutic options are limited [1,2]. Patients with IBD have increased intestinal permeability, barrier disfunction of the epithelium and cumulative exposure to antigens leading to activation of the immune system, production of pro-inflammatory cytokines and reactive oxygen species resultant in intestinal mucosa lesions [4].

Experimental models of IBD supply considerable information on the pathogenesis of this illness, regardless of they don't fully reflect the complexity of the human disease and has several disadvantages they represent an important tool in testing new strategies of treatment, since they mimic the features of the disease [6,7]. TNBS model, particularly, is a commonly used model of IBD to study the pathogenesis of colitis since its capable of reproduce CD in humans once promotes a Th1 immune response with characteristic chronic transmural colitis [6,83,94]. Additionally, TNBS model can mimic acute and chronic stages of IBD, through a simple and short experiment with reproducible and long-lasting colonic damage accompanied by inflammatory cell infiltration and ulcers [98,99]. The experimental model with TNBS consists in the induction of intestinal inflammation by a chemical process in which colitis is induced by rectal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS), the haptenating substance, in an ethanol solution, leads to deregulation of the mucosal immune system [100,101]. Thus the main aim of this project is to develop a preliminary study towards a novel chronic experimental model to study IBD in order to support a future new model of chronic induced colitis in rodents using TNBS.

In this study, it was used a chronic chemical model of colitis induction based on the described by Morris et al. [8]. Since the protocols of the TNBS-induced colitis model are not standardized [12], the experimental protocol was performed, with six independent groups of TNBS-induced colitis and consequently developed and monitored under the same specific conditions, however sacrificed on day 7 of each week after instillation, during a period of 6 weeks. The mice were evaluated taking into account parameters like clinical symptoms such as body weight, stool consistency, anus appearance and colon length and biochemical markers such as ALP, urea, creatinine, ALT, fecal hemoglobin, TNF-α and IL-10. TNBS-induced chronic colitis model was developed with the main objective to providing a chronic pattern of intestinal inflammation disease.

Regarding monitoring of clinical signs, TNBS group presented an alteration of intestinal motility characterized by diarrhea or soft stools, edema of the anus and moderate morbidity, while ethanol and sham groups remained without any alterations. The manifestations became more exacerbated at week 2 after induction and the effect maintain with more light signs in the next weeks. The TNBS 1 group presented moderate morbidity, the TNBS 2 group revealed high morbidity and the TNBS 4 group onwards presented mild morbidity. The sham group remained without any alterations. These clinical manifestations in the TNBS groups were expected and compatible with a correct chronic induction of CD [97,102].

The peak of clinical signs was also confirmed by a slight progressive increase in body weight in the entire experimental procedure with around 13.32 ± 2.7 % of weight gain, consistent with other research groups [97,102,103]. Three weeks after the initial

induction day, all TNBS groups presented a decrease in body weight, demonstrating that sick mice became weaker, with recovery body weight in the next weeks. Regarding control groups, they presented a considerable increase in body weight with a gain of 12.74 ± 2.3% ethanol group and 13.08 ± 2.4% sham group of its initial weight. In fact, they were fasted for 12 hours since the day before of the induction day. After induction, they woke up from anesthesia, felt healthy and prepared to eat, unlike the other groups with experimental colitis, thus justifying the increase.

TNBS-induced colitis promotes a reduction in the colon length compared to the sham groups [103,104]. In our study TNBS-induced colitis effect colon length on week 2, after that, the colon length significantly recovered TNBS effects, but in the following weeks the mice maintaining the effects on the colon length, suggesting that a exacerbated damage manifested on week 2 occurs, with maintaining effects from week 4 until the end of the experiment. In this study, mice without TNBS-induced colitis showed a normal colon length with approximately 10 cm, however we verified that TNBS-induced chronic colitis promotes a reduction of 1,5 cm in the colon length compared with sham group and, however, there was no significant variance between the TNBS groups and the sham group, although consistent with the findings from other research groups [103,104].

Regarding fecal hemoglobin it is expected to have higher values in the TNBS groups as opposed to the control group. The fecal hemoglobin was evaluated as an index of hemorrhagic focus in the damage colonic tissue and allow the detention of bleeding lesions for the diagnosis of several colorectal diseases with active inflammation. [105,106].The fecal hemoglobin also confirmed the maintain of TNBS-induced chronic colitis effect from 4 week onwards, since we verified a significantly increase of around 4 µmol Hg/g feces until week 6, evidence the presence of hemorrhagic ulcers. The control groups presented residual concentrations of fecal hemoglobin.

The ALP concentration on blood is a crucial marker regularly measured due to his essential role in intestinal homeostasis. [107,108]. ALP is expressed on the surface of enterocytes and is responsible for mucosal defense, promoting the interaction between the toll-like receptor-4 in the intestinal mucosa and the lipopolysaccharides derived from bacterial flora [109]. TNBS groups presented the highest values of serum total ALP concentration comparing with the control groups and increased ALP values were maintained from the fourth week onwards. The ALP concentrations detected for control groups where decreased comparing with the TNBS groups suggesting that the increased levels observed in TNBS groups where due to intestinal lesion inducted in this study. By his side, the ethanol has the facility to induce an intestinal permeability alteration origin colon injury. The ALP blood concentration results from group ethanol prove this damage since presented a slight increase of ALP compared with sham group. These our results are consistent with other studies, since higher serum ALP levels are confirmed in the chronic intestinal inflammation model induced by TNBS [110].

Cytokines are molecules involved in signal emission between cells during the triggering of immune responses. Cytokines are crucial for fighting infections and other immune responses. Pro-inflammatory cytokines work by promoting the inflammatory process, ensuring that reactions occur, and the initial insult is eliminated. Anti-inflammatory cytokines, by his side, act as a brake on this process, preventing an exacerbated response and possibly producing undesirable effects of the inflammation itself and the healing process [111]. TNF-α is a pro-inflammatory cytokine produced during the innate immune response of IBD [112]. TNF-α is associated to the pathogenesis of colitis,

since is increased in inflamed colon tissue [113,114]. Our obtained results confirm that the used TNBS induced colitis model promotes a significantly increase in the levels of TNF-α in the colon. Specifically, these cytokines registered a high level at week 3 and maintain these high values exacerbated until week 6 indicating an eventual onset of the chronic phase of this inflammatory disease. IL-10 plays a central role in the mucosal immune system by inhibiting proinflammatory cytokine synthesis and antigen presentation, and at the same time relieves intestinal inflammation [115].The presence of anti-inflammatory cytokines, as IL-10 was measured parallelly and the obtained data contrarily of what was expected allows concluding that TBNS-induced colitis promotes increased production of IL-10, observed after week 3. These results are congruent with the hypothesis that the immune system in presence of a chronic inflammatory insult tent to dispel the disease, balance values of biochemical markers of inflammation and consequently reestablish homeostasis [116,117].

Extraintestinal manifestations can involve almost every organ system and some of the most frequently involved organs are the liver and kidney [118,119]. Regarding to the urea and creatine evaluation, biomarkers of renal damage, the TNBS induced colitis doesn't present considerable alterations comparing with control groups. The ALT evaluation related with the hepatic function presented a significantly alteration in TNBS induced colitis values comparing with control groups. The high values of ALT were detected after week 4, remaining until the end of the experiment. Concerning our results TNBS-induced colitis suggests alterations in the liver but no significant damages in renal function.

Regarding the surveillance rate in our study we observed a surveillance decrease in the two first week of the procedure of approximately 50%, followed by a conservation in the levels obtained until the end of the study. These results are coincident with the peak of the disease. On the other side until the end of the experiment mice survival maintains indicate stability and adaptability of animals to disease as in the chronic pattern associated with this model.

In our study, a TNBS-induced colitis model was monitored for 6 weeks, since we wanted to achieve a chronic pattern of induced colitis and identify the week in which the damage become chronic. Manifestations became maximal on day 4 after induction and the effect began to decline from that day. Clinical manifestations of chronic colitis usually peak within 2 weeks and may be followed by partial recovery or death. These were expected and compatible with the correct induction of colitis [120-123]. Indeed, all clinical signs, biochemical markers, histopathological analysis and concentrations of pro- and anti-inflammatory cytokines, under evaluation, corroborate that the week from which a chronic illness pattern is observed is week 4 after the induction. Possibly, because some mice died during the early days of the study, no resisting to aggravation of the disease in its acute phase, while the remaining mice resisted and progressed to chronic phase of the disease, showing the same symptoms but more lightly. These preliminary data allow concluding that TNBS-induced chronic colitis should be developed in 4 weeks, providing a chronic intestinal inflammation model.

Actually, there is no knowledge of a cure, so the treatment of IBD focuses on the use of drugs that decrease the inflammatory process and induce remission of the disease, in addition current therapies are not unanimously effective and result in various side effects [3,124]. In the last decade, the treatment of IBD has evolved considerably, with the appearance of several drugs directed to block specific inflammatory chain components sparking researchers' interest in investigating the different drugs targeted

at a specific goal [25-27]. With this interest arises importance to study animal models of disease. Our study allowed create a TNBS-induced chronic colitis model with the main objective to provide a chronic pattern of intestinal inflammation disease to future pharmacological modulation with drugs like EPO, TDZD-8 and hemin.

Chapter 6 – Conclusion

Experimental animal models have been developed and used for preclinical studies since they have contributed greatly to important advances in our current understanding of the immunological, pathological, and physiological features of chronic intestinal inflammation [83,91]. This animal model of colitis used in our study is an efficient method, since present clinical manifestations similar to those observed in human IBD and are capable of mimic the pattern of inflammation in human, producing a rapid, reliable and reproducible disease [6,83,94]. Animal model conclusions may provide vision into potential therapeutic approaches to ameliorate the inflammation and to minimize the morbidity and mortality associated with IBD [12,91,125]. However, careful attention may be required to translate animal studies to clinical settings by ensuring that both safety and efficacy can be achieved and robust clinical trials providing such potential benefits must be recommended before the use of these drugs for the management of IBD. Once the protocols of the TNBS-induced colitis model are not standardized, variability in the results in preclinical studies, due to several conditions such as type of induction method, administered doses and treatment period, difficult the translation of the data for the clinical practice, as well as the degree of disease and time required to produce the injury may vary between laboratories [84,85].

In the chronic TNBS-induced colitis model, colonic inflammation is induced in susceptible strains of mice, by intrarectal administrations of the haptenating substance, TNBS, in increasing doses, together with ethanol [8]. In our study, the development of a TNBS-induced colitis model was essential, as well as the standardization and validation of the induction method. Several conditions were tested to achieve a standard induction method, such as the dose of TNBS, the depth of TNBS administration, the time point for model evaluation, and the concentration of ethanol, as TNBS vehicle. The advantage of chronic models compared to acute models is that the latter may provide only limited information about the pathogenesis of human IBDs, as the chemical injury to the epithelial barrier leading to self-limiting inflammation rather than to chronic disease [125].

The main objective established for this study was achieved. During this experiment, it was possible to develop a preliminary model of TNBS-induced chronic colitis. Throughout the induction method were evaluated and monitored several parameters, such as clinical signs, biochemical markers and concentration of pro- and anti-inflammatory cytokines. All parameters under evaluation corroborated that the damage became chronic 4 weeks after the induction with TNBS 1% instillations. So, TNBS-induced colitis was developed in 4 weeks, providing a chronic intestinal inflammation model. This model exhibits many of the pathological, molecular and immunological features of human colitis [8,126]. Additionally, the validation of this animal model is truly relevant to the scientific community, since there is no standard practice in the induction of colitis by TNBS. TNBS model can be a useful tool to study new metabolic pathways and new pharmacological approaches, consequently, facilitate a more effective and selective treatment than the currently known [127]. Supplementary experiments in the same research group were conducted in order to corroborate these findings. Upon these results, it is possible to propose a few topics for future development. Firstly,

analyze the data of histopathologic analysis of the bowel in order to assess the microscopic severity of colitis, allowing the evaluation of the extent of mucosal lesions in the collected tissues. Additionally, determine the concentration of IL-1β, a cytokine with a fundamental role in the pro-inflammatory immune response [128] and lastly, determine the myeloperoxidase activity, a specific biomarker of colonic oxidative stress [129].

In preclinical studies developed by our research group previously, was used 100 µL of 2.5% TNBS in 50% ethanol to induce an acute TNBS-induced colitis model as a result of which the maximal damage was manifested on day 4. Furthermore, was observed a reduction of the inflammation associated with IBD after administration of erythropoietin, thiadiazolidinone-8, or hemin [25-27].

Actually, and additional to findings of this work our research group propose to evaluate if all tested drugs previously in the acute model significantly inhibit chronic inflammatory response in this experimental chronic colitis model and if the efficacy and safety of these drugs are similar to comparing acute and chronic conditions. Some other interesting and promising examples of theoretically new strategies in IBD treatment include clarify the relevance of some changes in the drug delivery system and observe witch results that changes could promote in the therapeutic effect since topical application by rectal administration is eventually a good strategy to obtain a more effective and selective treatment with less adverse reactions.

Chapter 7 – References

1. Mowat, C., Cole, A., Windsor, A., Ahmad, T., Arnott, I., Driscoll, R., Bloom, S. (2011). Guidelines for the management of inflammatory bowel disease in adults. Gut, 60(5), 571–607. https://doi.org/10.1136/gut.2010.224154
2. Pawar, P., Gilda, S., Sharma, S., Jagtap, S., Paradkar, A., Mahadik, K., Ranjekar, P., Harsulkar, A. (2011). Rectal gel application of Withania somnifera root extract expounds antiinflammatory and mucorestorative activity in TNBS-induced Inflammatory Bowel Disease. BMC Complementary and Alternative Medicine, 11 (34), 1-9.
3. Zhang, L., Zhang, Y., Zhong, W., Di, C., Lin, X., Xia, Z. (2014). Heme oxygenase-1 ameliorates dextran sulfate sodium induced acute murine colitis by regulating Th17/Treg cell. The Journal of Biological Chemistry, 289 (39), 26847–26858.
4. Hanauer, S. B. (2006). Inflammatory Bowel Disease: Epidemiology, Pathogenesis, and Therapeutic Opportunities. Inflamm Bowel Dis, 12(January), 3–9.
5. Pithadia, A. B., & Jain, S. (2011). Treatment of inflammatory bowel disease (IBD). Pharmacological Reports, 63(3), 629–642. https://doi.org/10.1016/S1734-1140(11)70575-8
6. Randhawa, P. K., Singh, K., Singh, N., & Jaggi, A. S. (2014). A review on chemical-induced inflammatory bowel disease models in rodents. Korean Journal of Physiology and Pharmacology, 18(4), 279–288. https://doi.org/10.4196/kjpp.2014.18.4.279
7. Wirtz, S., & Neurath, M. F. (2007). Mouse models of inflammatory bowel disease. Advanced Drug Delivery Reviews, 59(11), 1073–1083. https://doi.org/10.1016/j.addr.2007.07.003
8. Morris, G. P., Beck, P. L., Herridge, M. S., Depew, W. T., Szewczuk, M. R., & Wallace, J. L. (1989). Hapten-Induced Model of Chronic Inflammation and Ulceration in the Rat Colon. Gastroenterology, 96(2), 795–803. https://doi.org/10.1016/S0016-5085(89)80079-4
9. Corazza, N., Eichenberger, S., Eugster, H.P., Mueller, C. (1999). Nonlymphocyte-Derived Tumor Necrosis Factor Is Required for Induction of Colitis in Recombination Activating Gene (Rag)2−/− Mice upon Transfer of Cd4+Cd45rbhi T Cells. J Exp Med, 190 (10), 1479-1792.
10. Seamons, A., Treuting, P.M., Brabb, T., Maggio-Price, L. (2013). Characterization of Dextran Sodium Sulfate-Induced Inflammation and Colonic Tumorigenesis in Smad3−/− Mice with Dysregulated TGFβ. PLoS One, 8 (11), e79182.
11. Lamb, K., Zhong, F., Gebhart, G., Bielefeldt, K. (2006). Experimental colitis in mice and sensitization of converging visceral and somatic afferent pathways. American Journal of Physiology - Gastrointestinal and Liver Physiology, 290, 451–457
12. Qin, H., Wu, J., Tong, X., Sung, J., Xu, H., Bian, Z. (2011). Systematic review of animal models of post-infectious/post-inflammatory irritable bowel syndrome. Journal of Gastroenterology, 46, 164–174.

13. Fuss, I., Neutrath, M., Boirivant, M., Klein, J., de la Motte, C., Strong, S., Fiocchi, C., Strober, W. (1996). Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-γ, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. Journal of Immunology, 157, 1261–1270
14. Abraham, C., & Cho, J. H. (2009). Inflammatory Bowel Disease. The New England Journal of Medicine, 2066–2078. https://doi.org/10.1056/NEJMra0804647
15. Kaser, A.; Zeissig, S.; Blumberg, R.S. Inflammatory bowel disease. Annu. Rev. Immunol. 2010, 28, 573–21.doi:10.1146/annurev-immunol-030409-101225.
16. Sartor, R. B. (2006). Mechanisms of disease: Pathogenesis of Crohn's disease and ulcerative colitis. Nature Clinical Practice Gastroenterology and Hepatology, 3(7), 390–407. https://doi.org/10.1038/ncpgasthep0528
17. Strober, W., Fuss, I., & Mannon, P. (2007). The fundamental basis of inflammatory bowel disease. Journal of Clinical Investigation, 117(3), 514–521. https://doi.org/10.1172/JCI30587.514
18. Azevedo, F., Magro, F., Portela, F., Lago, P., Deus, J., Cotter, J., Lopes, H. (2008). Estimating the prevalence of inflammatory bowel disease in Portugal using a pharmaco-epidemiological approach. Pharmacoepidemiology and Drug Safety, (March), 499–510. https://doi.org/10.1002/pds
19. Bernstein, C., Eliakim, A., Fedail, S., Fried, M., Gearry, R., Goh, K.-L., LeMair, A. (2015). World Gastroenterology Organisation Global Guidelines Inflammatory Bowel Disease. World Gastroenterology Organisation Global Guidelines, (August), 36. https://doi.org/10.1055/s-2004-826083
20. Bernstein, N., Fried, M., Eliakim, A., Fedail, S., Gearry, R., Goh, K.L., Hamid, S., Khan, A.G., Khalif, I., Ng, S.C, Ouyang, Q., Rey, J.F., Sood, A., Steinwurz, F., Watermeyer, G., LeMair, A. (2015). Inflammatory bowel disease: a global perspective. World Gastroenterology Organisation Global Guidelines.
21. Bernstein, C. N. (2015). Treatment of IBD: Where we are and where we are going. American Journal of Gastroenterology, 110(1), 114–126. https://doi.org/10.1038/ajg.2014.357
22. Mayer, L. (2010). Evolving paradigms in the pathogenesis of IBD. Journal of Gastroenterology, 45, 9–16.
23. Loftus, E.V., Sandborn, W.J. (2002). Epidemiology of inflammatory bowel disease. Gastroenterol Clin North Am, 31, 1–20.
24. Loftus, E. (2004). Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. Gastroenterology, 126, 1504–1517.
25. Mateus, V., Rocha, J., Alves, P., Mota-Filipe, H.; Sepodes, B., Pinto, R.M. Anti-inflammatory effect of erythropoietin in the TNBS-induced colitis. Basic Clin. Pharmacol. Toxicol. 2017, 120, 138–145. doi:10.1111/bcpt.12663.
26. Mateus, V., Rocha, J., Alves, P.; Mota-Filipe, H., Sepodes, B.; Pinto, R. Thiadiazolidinone-8 ameliorates inflammation associated with experimental colitis in mice. Pharmacology 2018, 101, 35–42. doi:10.1159/000471808.
27. Mateus, V., Rocha, J., Mota-Filipe, H., Sepodes, B., Pinto, R. Hemin reduces inflammation associated with TNBS-induced colitis. Clin. Exp. Gastroenterol. 2018, 11, 325–334. doi:10.2147/CEG.S166197.

28. Rocha, J., Eduardo-Figueira, M., Barateiro, A., Fernandes, A., Brites, D., Bronze, R., Duarte, C.M., Serra, A.T., Pinto, R.; Freitas, M., et al. Anti-Inflammatory effect of rosmarinic acid and an extract of Rosmarinus officinalis in rat models of local and systemic inflammation. Basic Clin. Pharmacol. Toxicol. 2015, 116, 398– 413. doi:10.1111/bcpt.12335.
29. Direito, R., Rocha, J., Lima, A., Gonçalves, M.M., Duarte, M.P., Mateus, V., Sousa, C.; Fernandes, A.; Pinto, R.; Ferreira, R.B.; et al. Reduction of inflammation and colon injury by a spearmint phenolic extract in experimental bowel disease in mice. Medicines 2019, 6, 65. doi:10.3390/medicines6020065.
30. Direito, R., Lima, A., Rocha, J., Ferreira, B.F., Mota, J., Rebelo, P., Fernandes, A., Pinto, R., Alves, P., Bronze, R., et al. Dyospiros kaki phenolics inhibit colitis and colon cancer cell proliferation, but not gelatinase activities. J. Nutr. Biochem. 2017, 46, 100–108. doi:10.1016/j.jnutbio.2017.03.002.
31. Kornbluth, A., Sachar, D.B. (2004). Ulcerative practice guidelines in adults (update): American College of Gastroenterology, Practice Parameters Committee. Am J Gastroenterol, 99, 1371–1385.
32. Kornbluth, A., Sachar, D.B. (2010). Ulcerative colitis practice guidelines in adults: American College of Gastroenterology, Practice Parameters Committee. Am J Gastroenterol, 105 (3), 501-523.
33. Doherty, G.A., Cheifetz, A.S. (2009). Management of acute severe ulcerative colitis. Expert Rev Gastroenterol Hepatol, 3 (4), 395-405.
34. Dignass, A., Eliakim, R., Magro, F., Maaser, C., Chowers, Y., Geboes, K., Mantzaris, G., Reinisch, W., Colombel, J., Vermeire, S., Travis, S., Lindsay, J.O., Assche, G.V. (2012). Second European evidence-based consensus on the diagnosis and management of ulcerative colitis - Part 1: Definitions and diagnosis. Journal of Crohn's and Colitis, 6, 965–990.
35. Wells, B. G., Dipiro, J. T., Schwinghammer, T. L., & Dipiro, C. V. (2009). Pharmacotherapy Handbook (7th ed.). The McGraw-Hill Companies.
36. Wells, B. G., DiPiro, J. T., Schwinghammer, T. L., & DiPiro, C. V. (2015). Pharmacotherapy Handbook. In Pharmacotherapy Handbook (9th ed.). McGraw Hill Education
37. Y.M. Michel, L., & Balligand, J.-L. (2015). Inflammatory Bowel Disease: Pathophysiology and Current Therapeutic Approaches. Handbook of Experimental Pharmacology, (January), 251–263. https://doi.org/10.1007/164
38. Silverberg, M., Satsangi, J., Ahmad, T., Arnott, I.D., Bernstein, C.N., Brant, S.R., Caprilli, R., Colombel, J.F., Gasche, C., Geboes, K., Jewell, D.P., Karban, A., Loftus, E.V.Jr., Peña, A.S., Riddell, R.H., Sachar, D.B., Schreiber, S., Steinhart, A.H., Targan, S.R., Vermeire, S., Warren, B.F. (2005). Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Canadian Journal of Gastroenterology, 19 (A), 5-36.
39. Satsangi, J., Silverberg, M., Vermeire, S., Colombel, J.F. (2006). The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut, 55, 749-753.
40. Freeman, H.J. (2008). Use of the Crohn's disease activity index in clinical trials of biological agents. World J Gastroenterol, 14 (26), 4127-4130.
41. Collins, P., Rhodes, J. (2006). Ulcerative colitis: diagnosis and management. BMJ, 333 (7563), 340-343.

42. Burisch J, Jess T, Martinato M, Lakatos PL. The burden of inflammatory bowel disease in Europe. J Crohn's Colitis. 2013;7:322-337.
43. Thoreson, R., Cullen, J.J. (2007). Pathophysiology of inflammatory bowel disease: an overview. Surg Clin North Am, 87 (3), 575-585.
44. Dias, C. C., Santiago, M., Correia, L., Portela, F., Ministro, P., Lago, P., Magro, F. (2019). Hospitalization trends of the Inflammatory Bowel Disease landscape: A nationwide overview of 16 years. Digestive and Liver Disease. https://doi.org/10.1016/j.dld.2019.01.016
45. Ng, S. C., Shi, H. Y., Hamidi, N., Underwood, F. E., Tang, W., Benchimol, E. I., Kaplan, G. G. (2017). Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. The Lancet, 390(10114), 2769–2778. https://doi.org/10.1016/S0140-6736(17)32448-0
46. Perl, D., Fogarty, U., Harpaz, N., Sachar, D.B. (2004). Bacterial–metal interactions: the potential role of aluminum and other trace elements in the etiology of Crohn's disease. Inflammatory Bowel Disease, 10, 881–883.
47. Lakatos, P.L. (2009). Environmental factors affecting inflammatory bowel disease: have we made progress? Dig Dis, 27 (3), 215-225.
48. Birrenbach, T., Bocker, U. (2004). Inflammatory bowel disease and smoking: a review of epidemiology, pathophysiology, and therapeutic implications. Inflammatory Bowel Disease, 10, 848–859.
49. Cosnes, J., Gowerrousseau, C., Seksik, P., & Cortot, A. (2011). Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology, 140(6), 1785-1794.e4. https://doi.org/10.1053/j.gastro.2011.01.055
50. Cosnes, J. (2004). Tobacco and IBD: relevance in the understanding of disease mechanisms and clinical practice. Best Practice & Research Clinical Gastroenterology, 18, 481–496.
51. Baumgart, D. C., & Sandborn, W. J. (2012). Crohn's disease. The Lancet, 380(9853), 1590–1605. https://doi.org/10.1016/S0140-6736(12)60026-9
52. Baumgart, D.C., Carding, S.R. (2007). Inflammatory bowel disease: cause and immunobiology. Lancet, 369 (9573), 1627-1640.
53. Berg, D., Zhang, J., Weinstock, J.V., Ismail, H.F., Earle, K.A., Alila, H., Pamukcu, R., Moore, S., Lynch, R.G. (2002). Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology, 123, 1527–1542.
54. Foersch, S., Waldner, M. J., & Neurath, M. F. (2013). Innate and adaptive immunity in inflammatory bowel diseases. Digestive Diseases, 31(3–4), 317–320. https://doi.org/10.1159/000354685
55. Valatas, V., Vakas, M., Kolios, G. (2013). The value of experimental models of colitis in predicting efficacy of biological therapies for inflammatory bowel diseases. Am J Physiol Gastrointest Liver Physiol, 305, 763–785.
56. Silva, F. A. R., Rodrigues, B. L., Ayrizono, M. D. L. S., & Leal, R. F. (2016). The Immunological Basis of Inflammatory Bowel Disease. Gastroenterology Research and Practice, 2016. https://doi.org/10.1155/2016/2097274
57. Yadav, V., Varum, F., Bravo, R., Furrer, E., Bojic, D., & Basit, A. W. (2016). Inflammatory bowel disease: exploring gut pathophysiology for novel

therapeutic targets. Translational Research, 176, 38–68. https://doi.org/10.1016/j.trsl.2016.04.009
58. Desreumaux, P., Brandt, E., Gambiez, L., Emilie, D., Geboes, K., Klein, O., Ectors, N., Cortot, A., Capron, M., Colombel, J. (1997). Distinct cytokine patterns in early and chronic ileal lesions of Crohn's disease. Gastroenterology, 113, 118–126.
59. Sandborn, W.J., Hanauer, S.B. (1999). Antitumor necrosis factor therapy for inflammatory bowel disease. A review of agents, pharmacology, clinical results, and safety. Inflamm Bowel Dis, 2, 119-133.
60. Sandborn, W.J., Hanauer, S.B. (2003). Systematic review: The pharmacokinetic profiles of oral mesalazine formulations and mesalazine pro-drugs used in the management of ulcerative colitis. Aliment Pharmacol Ther, 17, 29–42.
61. Ferguson, L. R., & Gentschew, L. (2014). Inflammatory bowel disease: Pathogenesis. Advanced Nutrition and Dietetics in Gastroenterology, 20(1), 169–179. https://doi.org/10.1002/9781118872796.ch3.12
62. Steinwurs F, Scheinberg MA. Diagnóstico sorológico de doença inflamatória intestinal (ASCA e ANCA): avaliação de 70 casos. GED 2001; 20:41-2.
63. Mackalski, B.A., Bernstein, C.N. (2006). New diagnostic imaging tools for inflammatory bowel disease. Gut, 55 (5), 733-741.
64. Nikolaus, S., Schreiber, S. (2007). Diagnostics of inflammatory bowel disease. Gastroenterology, 133 (5), 1670-1689.
65. Lichtenstein, G. R., Loftus, E. V., Isaacs, K. L., Regueiro, M. D., Gerson, L. B., & Sands, B. E. (2018). ACG Clinical Guideline: Management of Crohn's Disease in Adults. American Journal of Gastroenterology, 113(4), 481–517. https://doi.org/10.1038/ajg.2018.27
66. Aloi, M., Nuti, F., Stronati, L., Cucchiara, S. (2014). Advances in the medical management of paediatric IBD. Nature Reviews Gastroenterology & Hepatology, 11, 99-108.
67. Bamias, G., Arseneau, K. O., & Cominelli, F. (2017). Mouse models of inflammatory bowel disease for investigating mucosal immunity in the intestine. Current Opinion in Gastroenterology, 33(6), 411–416. https://doi.org/10.1097/MOG.0000000000000402
68. Zundler, S., Neurath, M.F. (2015). How will new and future therapies change our treatment of IBD? Expert Rev Clin Immunol, 1–4.
69. Engel, M. A., & Neurath, M. F. (2010). New pathophysiological insights and modern treatment of IBD. Journal of Gastroenterology, 45(6), 571–583. https://doi.org/10.1007/s00535-010-0219-3
70. Sandborn, W.J., Hanauer, S.B. (2003). Systematic review: The pharmacokinetic profiles of oral mesalazine formulations and mesalazine pro-drugs used in the management of ulcerative colitis. Aliment Pharmacol Ther, 17, 29–42.
71. Buning, C., Lochs, H. (2006). Conventional therapy for Crohn's disease. World J Gastroenterol, 12 (30), 4794-4806.
72. Barnes, P., Karin, M. (1997). Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases. New England Journal of Medicine, 336, 1066–1071.

73. Triantafillidis, J. K., Merikas, E., & Georgopoulos, F. (2011). Current and emerging drugs for the treatment of inflammatory bowel disease. Drug Design, Development and Therapy, 5, 185–210. https://doi.org/10.2147/DDDT.S11290
74. Hofer, K.N. (2003). Oral budesonide in the management of Crohn's disease. Ann Pharmacother, 37, 1457–1464.
75. Khan, K. J., Ullman, T. A., Ford, A. C., Abreu, M. T., Abadir, A., Marshall, J. K., Moayyedi, P. (2011). Antibiotic therapy in inflammatory bowel disease: A systematic review and meta-analysis. American Journal of Gastroenterology, 106(4), 661–673. https://doi.org/10.1038/ajg.2011.72
76. Aratari, A., Bossa, F., Cappello, M., Cassinotti, A., Chiriatti, A., Fiorino, G., Rugge, M. (2017). Safety of treatments for inflammatory bowel disease: Clinical practice guidelines of the Italian Group for the Study of Inflammatory Bowel Disease (IG-IBD). Digestive and Liver Disease, 49(4), 338–358. https://doi.org/10.1016/j.dld.2017.01.141
77. Thia, K. T., Mahadevan, U., Feagan, B. G., Wong, C., Cockeram, A., Bitton, A., Sandborn, W. J. (2009). Ciprofloxacin or metronidazole for the treatment of perianal fistulas in patients with Crohn's disease: A randomized, double-blind, placebo-controlled pilot study. Inflammatory Bowel Diseases, 15(1), 17–24. https://doi.org/10.1002/ibd.20608
78. Jess, T., Gamborg, M., Munkholm, P., Sorensen, T.I. (2007). Overall and cause-specific mortality in ulcerative colitis: meta-analysis of population-based inception cohort studies. Am J Gastroenterol, 102 (3), 609-617.
79. Catana, C. S., Magdas, C., Tabaran, F. A., Craciun, E. C., Deak, G., Magdas, V. A., Dumitrascu, D. L. (2018). Comparison of two models of inflammatory bowel disease in rats. Advances in Clinical and Experimental Medicine, 27(5), 599–607. https://doi.org/10.17219/acem/69134
80. Vandamme, T. F. (2015). Rodent models for human diseases. European Journal of Pharmacology, 759, 84–89. https://doi.org/10.1016/j.ejphar.2015.03.046
81. Gadaleta, R. M., Garcia-Irigoyen, O., & Moschetta, A. (2017). Exploration of Inflammatory Bowel Disease in Mice: Chemically Induced Murine Models of Inflammatory Bowel Disease (IBD). Current Protocols in Mouse Biology, 7(1), 13–28. https://doi.org/10.1002/cpmo.20
82. Bang, B., & Lichtenberger, L. M. (2016). Methods of inducing inflammatory bowel disease in mice. In Current Protocols in Pharmacology (Vol. 2016). https://doi.org/10.1002/0471141755.ph0558s47
83. Hibi, T., Ogata, H., Sakuraba, A. (2002). Animal models of inflammatory bowel disease. J Gastroenterol, 37, 409–417.
84. Mizoguchi, A. (2012). Animal Models of Inflammatory Bowel Disease. In Progress in Molecular Biology and Translational Science (1st ed., Vol. 105). https://doi.org/10.1016/B978-0-12-809468-6.00019-X
85. Mizoguchi, A., Mizoguchi, E. (2010). Animal models of IBD: linkage to human disease. Current Opinion in Pharmacology, 10, 578–587.
86. Bramhall, M., Flórez-Vargas, O., Stevens, R., Brass, A., Cruickshank, S. (2015). Quality of Methods Reporting in Animal Models of Colitis. Inflamm Bowel Dis, 21, 1248–1259.

87. Ohkusa, T. (1985). Production of experimental ulcerative colitis in hamsters by dextran sulfate sodium and a change of intestinal microflora. Japanese Journal of Gastroenterology, 82, 1327–1336
88. Okayasu, I., Hatekeyama, S., Yamada, M., Ohkusa, T., Inagaki, Y., Nakaya, R. (1990). A novel method in the induction of reliable experimental acute and ulcerative colitis in mice. Gastroenterology, 98, 694–702.
89. Perse, M., Cerar, A. (2012). Dextran sodium sulphate colitis mouse model: traps and tricks. J Biomed Biotechnol, 13.
90. Low, D., Nguyen, D.D., Mizoguchi, E. (2013). Animal models of ulcerative colitis and their application in drug research. Drug Des Devel Ther, 7, 1341–1357.
91. Murthy, S. (2006). In Vivo Models of Inflammation: Animal models of inflammatory bowel disease. Progress in Inflammation Research, 2, 137-174.
92. Jurjus, A.R., Khoury, N.N., Reimund, J.M. (2004). Animal models of inflammatory bowel disease. J Pharmacol Toxicol Methods, 50 (2), 81-92.
93. Linden, D.R., Chen, J.X., Gershon, M.D., Sharkey, K.A., Mawe, G.M. (2003). Serotonin availability is increased in mucosa of guinea pigs with TNBS induced colitis. Am J Physiol Gastrointest Liver Physiol, 285, 207–216.
94. Linden, D., Foley, K., McQuoid, C., Simpson, J., Sharkey, K.,Mawe, G. (2005). Serotonin transporter function and expression are reduced in mice with TNBS-induced colitis. Neurogastroenterology and Motility, 17, 565–574.
95. Antoniou, E., Margonis, G. A., Angelou, A., Pikouli, A., Argiri, P., Karavokyros, I., Pikoulis, E. (2016). The TNBS-induced colitis animal model: An overview. Annals of Medicine and Surgery, 11, 9–15. https://doi.org/10.1016/j.amsu.2016.07.019
96. Padua, D., Vu, J. P., Germano, P. M., & Pisegna, J. R. (2016). The Role of Neuropeptides in Mouse Models of Colitis. J Mol Neurosci, 59(2), 203–210. https://doi.org/10.1002/jmri.25711.PET/MRI
97. Motavallian-Naeini, A., Andalib, S., Rabbani, M., Mahzouni, P., Afsharipour, M., & Minaiyan, M. (2012). Validation and optimization of experimental colitis induction in rats using 2, 4, 6-trinitrobenzene sulfonic acid. Research in Pharmaceutical Sciences, 7(3), 159–169.
98. Dohi, T., Fujihashi, K. (2006). Type 1 and 2 T Helper Cell mediated Colitis. Curr Opin Gastroenterol, 22, 651–657.
99. Zheng, L., Gao, Z.Q., Wang, S.X. (2000). A chronic ulcerative colitis model in rats. World J Gastroenterol, 6, 150–152.
100. Menozzi, A., Pozzoli, C., Poli, E., Lazzaretti, M., Grandi, D., Coruzzi, G. (2006). Long-term study of TNBS-induced colitis in rats: Focus on mast cells. Inflamm Res, 55, 416–422.
101. Li, J.H., Yu, J.P., Yu, H.G., Xu, X.M., Yu, L.L., Liu, J., Luo, H.S. (2005). Melatonin reduces inflammatory injury through inhibiting NF-κB activation in rats with colitis. Mediat Inflamm, 4, 185–193.
102. Cuzzocrea, S., Mazzon, E., Paola, R., Patel, N., Genovese, T., Muia, C., Sarro, A., Thiemermann, C. (2004). Erythropoietin Reduces the Development of Experimental Inflammatory Bowel Disease. JPET, 311, 1272–1280
103. Majewska-Szczepanik, M., Góralska, M., Marcińska, K., Zemelka-Wiącek, M., Strzępa, A., Dorozyńska, I., & Szczepanik, M. (2012). Epicutaneous immunization with protein antigen TNP-Ig alleviates TNBS-

induced colitis in mice. Pharmacological Reports, 64(6), 1497–1504. https://doi.org/10.1016/S1734-1140(12)70947-7
104. Nayak, S. B., George, B. M., & Mishra, S. (2012). Abnormal length and position of the sigmoid colon and its clinical significance. Kathmandu University Medical Journal, 10(40), 95–97.
105. Hirata, I., Hoshimoto, M., Saito, O., Kayazawa, M., Nishikawa, T., Murano, M., Matsuse, R. (2007). Usefulness of fecal lactoferrin and hemoglobin in diagnosis of colorectal diseases. World Journal of Gastroenterology, 13(10), 1569–1574. https://doi.org/10.3748/wjg.v13.i10.1569
106. Mooiweer, E., Fidder, H.H., Siersema, P.D., Laheij, R.J., Oldenburg, B. (2014). Fecal hemoglobin and calprotectin are equally effective in identifying patients with inflammatory bowel disease with active endoscopic inflammation. Inflamm Bowel Dis, 20 (2), 307-314.
107. Moscandrew, M., Mahadevan, U., Kane, S. (2009). General health maintenance in IBD. Inflamm Bowel Dis, 15 (9), 1399-1409.
108. Tinnion, R., Embleton, N. (2012). How to use...alkaline phosphatase in neonatology. Archives of Disease in Childhood – Education and Practice Edition, 97 (4), 157-163, doi:10.1136/archdischild-2012-301633
109. Malo, M.S., Alam, S.N., Mostafa, G., Zeller, S.J., Johnson, P.V., Mohammad, N., Chen, K.T., Moss, A.K., Ramasamy, S., Faruqui, A., Hodin, S., Malo, P.S., Ebrahimi, F., Biswas, B., Narisawa, S., Millán, J.L., Warren, H.S., Kaplan, J.B., Kitts, C.L., Hohmann, E.L., Hodin, R.A. (2010). Intestinal alkaline phosphatase preserves the normal homeostasis of gut microbiota. Gut, 59, 1476-1484.
110. Luchini, A. C., Rodrigues-Orsi, P., Cestari, S. H., Seito, L. N., Witaicenis, A., Pellizzon, C. H., & Di Stasi, L. C. (2008). Intestinal anti-inflammatory activity of coumarin and 4-hydroxycoumarin in the trinitrobenzenesulphonic acid model of rat colitis. Biological and Pharmaceutical Bulletin, 31(7), 1343–1350. https://doi.org/10.1248/bpb.31.1343
111. Műzes, G., Molnár, B., Tulassay, Z., Sipos, F. (2012). Changes of the cytokine profile in inflammatory bowel diseases. World J Gastroenterol, 18 (41), 5848-5861.
112. Lee, T. W., & Fedorak, R. N. (2010). Tumor Necrosis Factor-α Monoclonal Antibodies in the Treatment of Inflammatory Bowel Disease: Clinical Practice Pharmacology. Gastroenterology Clinics of North America, 39(3), 543–557. https://doi.org/10.1016/j.gtc.2010.08.018
113. Nairz, M., Schroll, A., Moschen, A., Sonnweber, T., Theurl, M., Theurl, I., Taub, N., Jamnig, C., Neurauter, D., Huber, L., Tilg, H., Moser, P., Weiss, G. (2011). Erythropoietin Contrastingly Affects Bacterial Infection and Experimental Colitis by Inhibiting Nuclear Factor-kB-Inducible Immune Pathways. Immunity, 34, 61–74.
114. Paunovic, B., Deng, X., Khomenko, T., Ahluwalia, A., Tolstanova, G., Tarnawski, A., Szabo, S., Sandor, Z. (2011). Molecular mechanisms of basic fibroblast growth factor effect on healing of ulcerative colitis in rats. J Pharmacol Exp Ther, 339, 430–437.
115. Xiong, J., Lin, Y. H., Bi, L. H., Wang, J. De, Bai, Y., & Liu, S. De. (2013). Effects of Interleukin-4 or Interleukin-10 gene therapy on trinitrobenzenesulfonic

acid-induced murine colitis. BMC Gastroenterology, 13(1), 1–7. https://doi.org/10.1186/1471-230X-13-165
116. Leach, M., Davidson, N., Fort, M., Powrie F, Rennick DM.(1999) The role of IL-10 in inflammatory bowel disease: "of mice and men". Toxicol Pathol. 1999 Jan-Feb;27(1):123-33.
117. Marlow,G., Gent, D., and Ferguson, L.(2013) Why interleukin-10 supplementation does not work in Crohn's disease patients. World J Gastroenterol. 2013 Jul 7; 19(25): 3931–3941.Published online 2013 Jul 7. doi: 10.3748/wjg.v19.i25.3931
118. Rojas-Feria, M., Castro, M., Suárez, E., Ampuero, J.,Romero-Gómez, M. (2013). Hepatobiliary manifestations in inflammatory bowel disease: the gut, the drugs and the liver. World J Gastroenterol, 19 (42), 7327-7340.
119. Corica, D., Romano, C. (2015). Renal Involvement in Inflammatory Bowel Diseases. J Crohns Colitis, 29, 138.
120. Neurath, M.F. (2014). Cytokines in inflammatory bowel disease. Nat Rev Immunol, 14 (5), 329–342.
121. Neurath, M.F. (2014). New targets for mucosal healing and therapy in inflammatory bowel diseases. Mucosal Immunol, 7 (1), 6–19.
122. Neurath, M. F. (1995). Antibodies to interleukin 12 abrogate established experimental colitis in mice. Journal of Experimental Medicine, 182(5), 1281–1290. https://doi.org/10.1084/jem.182.5.1281
123. Neurath, Markus F. (2012). Animal models of inflammatory bowel diseases: Illuminating the pathogenesis of colitis, ileitis and cancer. Digestive Diseases, 30(SUPPL. 1), 91–94. https://doi.org/10.1159/000341131
124. Wilhelm, S. M., & Love, B. L. (2017). Management of patients with inflammatory bowel disease: current and future treatments. Clinical Pharmacist, 9(3). https://doi.org/10.1211/CP.2017.20202316
125. Wirtz, S., Popp, V., Kindermann, M., Gerlach, K., Weigmann, B., Fichtner-Feigl, S., & Neurath, M. F. (2017). Chemically induced mouse models of acute and chronic intestinal inflammation. Nature Protocols, 12(7), 1295–1309. https://doi.org/10.1038/nprot.2017.044
126. Whittle, B., Varga, C., Pósa, A., Molnár, A., Collin, M., Thiemermann, C. (2006). Reduction of experimental colitis in the rat by inhibitors of glycogen synthase kinase-3β. British Journal of Pharmacology, 147, 575–582.
127. Tomasello, G., Sinagra, E., Raimondo, D., Palumbo, V.D., Puleio, R., Cottone, M., Damiani, P., Traina, G., Abruzzo, A., Damiani, F., Buscemi, S., Noto, M., Lo Monte, A.I. (2015). Validation of a modified model of TNBS-induced colitis in rats. How to induce a chemical colitis in rats. Acta Biomed, 86 (1), 92-96.
128. Bilsborough, J., Targan, S. R., & Snapper, S. B. (2016). Therapeutic Targets in Inflammatory Bowel Disease: Current and Future. The American Journal of Gastroenterology Supplements, 3(3), 27–37. https://doi.org/10.1038/ajgsup.2016.18
129. Hansberry, D. R., Shah, K., Agarwal, P., & Agarwal, N. (2017). Fecal Myeloperoxidase as a Biomarker for Inflammatory Bowel Disease. Cureus, 9(Cd), 1–7. https://doi.org/10.7759/cureus.1004